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Hold to nothing too violently. Every fool stands convinced; and everyone convinced is a fool; and the faultier a man's judgment, the firmer his conviction; even when the proof is on your side, it is well to make concession; for your reasons are not unknown.

—Baltasar Gracian
A Truthtelling Manual and the Art of Worldly Wisdom

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Errata, Volume 31

Page 38, table 3, column 5: second line under 'Total' for 9.58±.70 *read* 11.95±.40; *third line under 'Total', for* 11.95±.40 *read* 11.56±.31; *last line under 'Total', for* 11.56±.31 *read* 11.81±.52.

ENDOCRINOLOGY

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VOLUME 31 JULY, 1942 NUMBER 1

EFFECT OF EQUINE GONADOTROPIN ON TESTES OF HYPOPHYSECTOMIZED MONKEYS[1]

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NEW YORK CITY

EQUINE GONADOTROPIN (pregnant-mare-serum gonadotropin) is undoubtably the most potent gonad-stimulating substance which at present is available in quantities sufficient for extensive animal or clinical experimental work. When injected into females, it causes follicular growth and luteinization in hypophysectomized rodents (2, 5, 6, 8), and produces follicular growth in normal monkeys (1, 10). In hypophysectomized male rats and guinea pigs it maintains or restores spermatogenesis and stimulates the interstititial tissue and accessory reproductive organs (2, 7, 9). These gonadotropic effects can be maintained for long periods with continued treatment.

Effects of this gonadotropin on either normal or hypophysectomized male monkeys have not been reported. They would seem to be of interest, for the type of involution of the seminiferous tubules in monkeys is similar to that in the human male and differs from that occurring in rats—the form on which previous experimental work on the maintenance or restoration of the testes after hypophysectomy has been done almost exclusively. In rats after ablation of the hypophysis, mitoses in the spermatogonia are frequent for long periods, although sperm do not form. In mature monkeys approximately 30 days after the removal of the pituitary gland only Sertoli cells and an occasional basally placed spermatogonium are present in the seminiferous tubules (fig. 4, 6). The spermatogonia rarely show mitoses. Thus in monkeys, any therapy which restores sperm formation must not only carry to maturity the later generations of the sperm-forming cells but must also induce division in the spermatogonia. A similar condition also obtains in the completely involuted tubules of the testes in man. This might presumably make a restoration of sperm formation in monkeys (and man) more difficult than in rats.

[1] This work was aided by a grant from the Rockefeller Foundation.

Part of the material of this paper was presented in the Presidential Address given at the twenty-fourth annual meeting of the Association for the Study of Internal Secretions, New York, 1940.

* This paper was contributed for the Twenty-fifth Anniversary Number (June) of ENDOCRINOLOGY, but owing to circumstances beyond the control of the author it was not available in time to appear in that issue.

MATERIAL AND METHODS

Mature rhesus monkeys were used. Mature monkeys are essential for only in them do the testes show the pronounced structural changes stated above. Although the testes of immature monkeys decrease in size after hypophysectomy, nevertheless, the structure of their seminiferous tubules remains essentially unchanged and restorative effects would have to be judged largely from changes in the size of the tubules. To secure spermatogenesis in them, furthermore, would entail the induction of maturity.

Eight hypophysectomized rhesus monkeys were treated for varying periods with different dosages of two preparations of pregnant-mare-serum gonadotropin.[2] In some of the animals treatment was begun immediately after hypophysectomy; in others treatment was postponed until the seminiferous tubules had undergone partial or complete involution. Large biopsy specimens of the testes were taken at intervals. Since a study of sections of different parts of testes from autopsies reveals quite a uniform condition of the tubules throughout the gland, it is believed that the biopsies give a reliable picture of the condition obtaining throughout the entire organ.

Observations on control non-hypophysectomized animals have shown no deleterious effects of the biopsies on the testis except some occasional local fibrosis at the site of the wound. Adhesions between the visceral and parietal layers of the tunica vaginalis at the site of the biopsy occurred in some cases.

Measurements of the testes were taken when the biopsies were obtained. These were taken either with the testes completely exposed or by the more inaccurate methods of measurement through the scrotum. This latter method is sufficiently accurate, however, to reveal the major changes in testicular size which occur after hypophysectomy.

Complete serial sections of the pituitary capsule from each animal were made and carefully examined to check the completeness of the ablation of the anterior and posterior lobes. The weight and structure of the adrenals and thyroids was also determined. These supply excellent adjuvant evidence on the completeness of the ablation.

Since I have not published any note in regard to the procedure employed in recent years in ablating the hypophysis in monkeys, it seems justifiable at least to describe the general features of the operative procedure. The parapharyngeal approach has been found to be easily adaptable to monkeys. Westman and Jacobsohn (16) have briefly described this approach in rabbits. It has the great advantage over the oral approach, in that a sterile technique can be used. As good an exposure of the gland is secured as by the oral approach and trauma, while somewhat greater than in that approach, is not severe.

The insertion of a tracheal cannula is essential, for the breathing passages are entirely occluded by the retraction of the nasopharynx. After the insertion of the cannula, a longitudinal incision some 3 to 4 cm. in length is made on one side, caudal and somewhat medial to the angle of the mandible. The platysma and deeper fascia are divided and access to the region between the buccopharyngeal and prevertebral fascia is gained, leaving the structures in the carotid sheath, the stylohyoid and anterior belly of the digastric muscles intact. The buccopharyngeal and prevertebral fasciae are then separated cephalically to the base of the skull by blunt dissection. The tendinous insertion of the prevertebral muscles on the basisphenoid and superior part of the occipital bone is removed, thus exposing the occipito-sphenoid suture.

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[2] Heartiest thanks are expressed to Dr. Donald Wonder of the Cutter Laboratories, Berkeley, Calif. for a generous supply of Gonadin. It was assayed in Cole-Saunders units (C.-S.U.); one unit is stated by the manufacturers to be approximately equal to two international units.

This is an essential landmark. A tubular shaped canal some 5 mm. in diameter is then drilled with a dental burr through the sphenoid to the hypophyseal capsule. The canals tarts just cephalad to the suture superficially but departs from it in its deeper portion. After fully exposing the ventral surface of the hypophyseal capsule, this is incised and the gland cautiously removed piecemeal with a curette and suction. The field is kept dry and a complete view maintained at all times. Although during and also at the completion of the removal of the gland the lateral walls of the pituitary capsule are not visible, nevertheless, there is little uncertainty about the completeness of the removal. The diaphragma is clearly visible together with the severed end of the pituitary stalk. In order to destroy any small remnants of the gland which may remain, the capsule is routinely sponged with 5 per cent chromic acid. If there is seepage of blood or cerebrospinal fluid, a small piece of muscle is placed in the entrance to the capsule. The wound is closed with superficially placed silk sutures and with suture clips. The operation is facilitated and made more exact by using a binocular of long focus giving a magnification of 2 to 3 diameters.

Recovery is excellent, but after a few days careful watch has to be kept for hypoglycemic symptoms and appropriate measures taken if they occur.

Rate of involution of seminiferous tubules. As has been pointed out elsewhere (14) the tubules in rhesus monkeys show little change in structure during the first 10 days after hypophysectomy and the testes do not decrease appreciably in size during this period. The rate of involution thus is not nearly as rapid as in rats. At 20 days postoperatively, the testes show a marked decrease in size and are somewhat flabby. Motile sperm in the testes and epididymis are still present, however. Apparently most of the later generations of sperm-forming cells undergo maturation.

At 30 days, sperm may be present but they are in much reduced numbers and most of the tubules show no mitoses of the spermatogonia. Primary and secondary spermatocytes are rare. The preoperative condition and vigor of the animal apparently influence somewhat the rate of involution of the testes although quantitative data on this point are difficult to obtain.

RESULTS

Postponed treatments. In 5 animals injection of equine gonadotropin was not begun until postoperative periods of from 20 to 63 days had elapsed (table 1). In the shortest of these postoperative pretreatment periods (20 days, *monkey* 660, fig. 2), spermatocytes, spermatids and sperm were still abundant but mitoses in the spermatogonia were rare. A biopsy taken after 10 days of treatment (300 C.-S.U. per day) showed an increased number of mitoses in the spermatogonia but only a few spermatocytes and sperm (fig. 3). Apparently the spermatocytes present at the time treatment was begun had matured but adequate time had not elapsed for the formation of sperm from the division of spermatogonia. After 26 days of treatment, restoration of spermatogenesis had not progressed beyond that existing after 10 days of treatment (fig. 4). Some spermatocytes but no sperm were present.

In the two animals in which involution was well advanced (*monkey* 385) or was complete (*monkey* 597) at the time treatment was begun, the injections of gonadotropin produced definite but not pronounced activation of the seminiferous tubules (fig. 5, 6, 7, 8, 9). This repair did not progress sufficiently for sperm to be formed. In one of these animals treatment was continued for 40 days, and the restorative effect was not maintained but was succeeded by a further involution during the latter part of the treatment period.

Two other animals (*monkeys* 370, 371) received only 40 C.-S.U. per day. In neither of these were there any reparative effects and in fact the one in which involution of

TABLE 1. EFFECTS ON TESTES OF DAILY INJECTIONS OF PREGNANT-MARE-SERUM GONADOTROPIN IN HYPOPHYSECTOMIZED MALE RHESUS MONKEYS

In each animal a biopsy of one of the testes was taken at the time of hypophysectomy. These biopsies showed in every animal that the seminiferous tubules were large and were in active spermatogenesis with numerous sperm.

Monkey	Weight at Hypophysectomy	Treatment Begun				Duration and effect of Treatment
		Number days operated	Involution seminiferous tubules	Extract	Dosage, per day[1]	
	gm.					
370	5450	25	Complete	Antex	200 M.U.	28 days; no restoration; some further decrease in size of seminferous tubules
385	7760	29	Complete	Antex	500 M.U.	9 days; tubules enlarged with central cavities; a few mitoses
597	7100	63[2]	Complete	Gonadin	200 C.-S.U.	40 days; biopsies after 20, 30 and 40 days; at 20 days definite restorative effects; mitoses, and spermatocytes *I*; no further improvement and some regression by 40th day; animal had erections
371	5550	26	Almost complete	Antex	200 M.U.	10 days; further involution occurred
660	10550	20	Partial[3]	Gonadin	300 C.-S.U.	26 days; biopsies after 10 and 26 days; *at 10 days* an increase in mitoses in spermatogonia; no spermatocytes *I* or *II*; some sperm; *at 26 days* no further repair; no mitoses in spermatogonia; some spermatocytes *I*, *II*; no sperm
377	7780	0	None	Antex	300 M.U.	20 days; tubules active; pronounced but not complete maintenance
379	8650	0	None	Antex	500 M.U.	18 days; complete maintenance
670	9750	0	None	Gonadin	250 C.-S.U.	57 days; biopsies after 10, 20, 30 and 57 days; maintenance for 20 days was excellent; at 30 days tubules much smaller and without cavities but were some mitoses and sperm; at 56 days involution much more marked but not quite complete; no sperm or spermatids

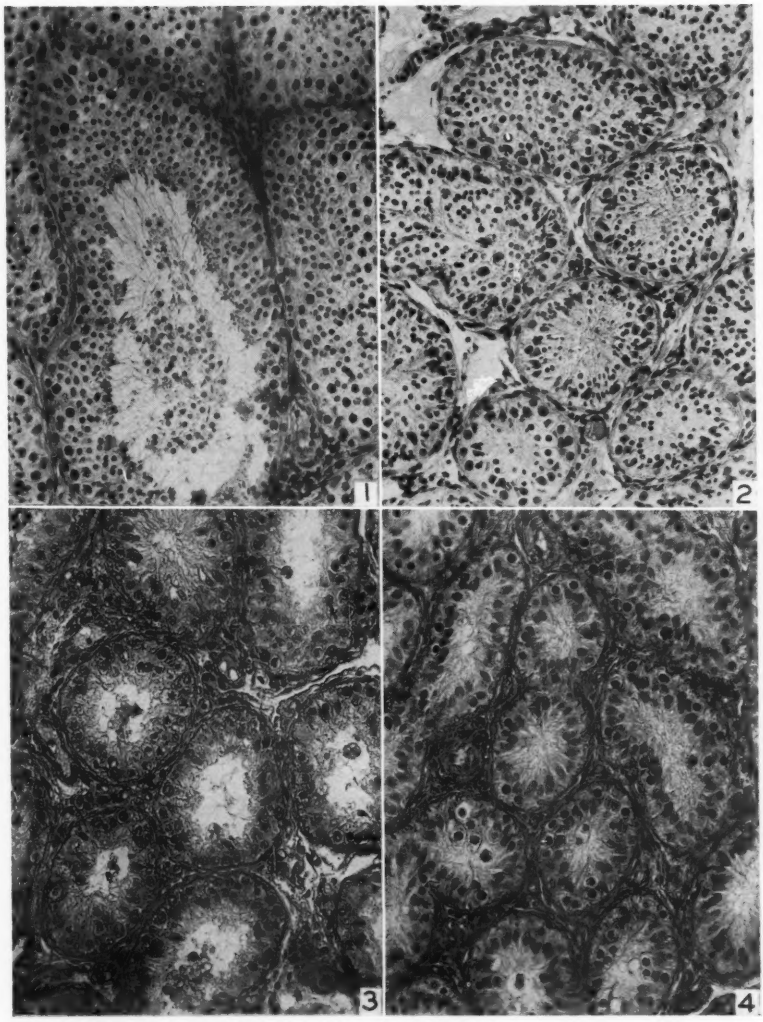
[1] One Cole-Saunders unit (C.-S.U.) is approximately equal to 5 Danish mouse units (M.U.). One C.-S.U. is stated by the manufacturers to be equal to 2 I.U.

[2] Androgen was given for the first 20 days after hypophysectomy. During the subsequent 43 days before gonadotropin was given there was complete involution.

[3] Mitoses in spermatogonia rare; a few spermatocytes, *I*; many spermatocytes, *II*, spermatids and sperm. Measurements of exposed testes in mm. are as follows: At hypophysectomy 40×30; after 20 days, (no treatment) 34×26; after 10 days treatment 36×28; after 26 days treatment 30×23. The slight increase in size after 10 days treatment is within the error of measurement whereas the subsequent decrease is certainly greater than the error of measurement.

the testes was incomplete showed further regression. The dosage was obviously too small to initiate restorative changes.

Thus, in no case in animals receiving the postponed treatments did the injections



ALL PHOTOGRAPHS were taken with 4 mm. objective and 12.5 ocular. They are reproduced at a uniform magnification in all figures.

Fig. 1. TESTIS OF *monkey* 660. Biopsy taken at the time of hypophysectomy. Fig. 2. TESTIS OF *monkey* 660. Biopsy 20 days later during which period no treatment had been given. Treatment was then begun.

Fig. 3. TESTIS OF *monkey* 660. Biopsy after 10-day treatment with 300 C.-S.U. of equine gonadotropin per day. Fig. 4. TESTIS OF *monkey* 660. Biopsy after 26-day treatment with 300 C.-S.U. of equine gonadotropin per day.

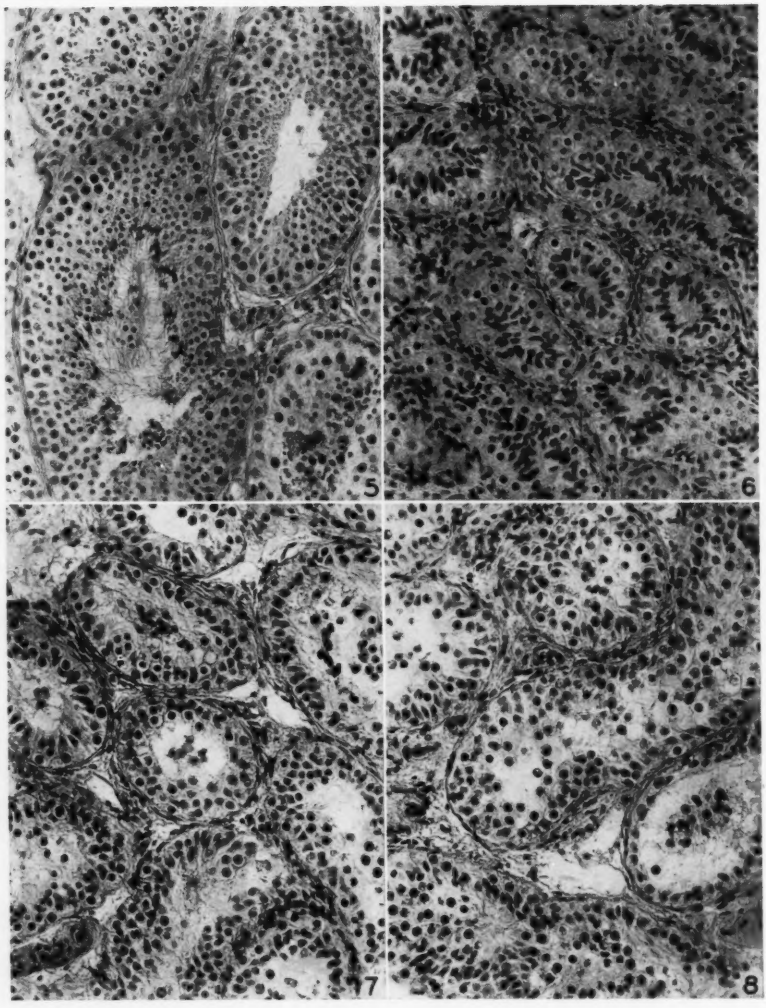


Fig. 5. Testis of *monkey* 597. Biopsy taken at the time of hypophysectomy. Fig. 6. Testis of *monkey* 597. Biopsy taken 63 days after hypophysectomy. During the first 20 days androgen was injected but in the next 43 days no treatment was given and there was complete involution. Treatment with equine gonadotropin was then started.

Fig. 7. Testis of *monkey* 597. Biopsy after 20 day-treatment with 200 C.-S.U. of equine gonadotropin per day. Fig. 8. Testis of *monkey* 597. Another area of biopsy shown in figure 7. (For biopsy taken 20 days later see fig. 9.)

cause the formation of sperm. Restorative effects were evident early in the treatment but they did not persist when the injections were continued over an extended period. The results were thus disappointing and definitely inferior to those reported in hypophysectomized rats and guinea pigs.

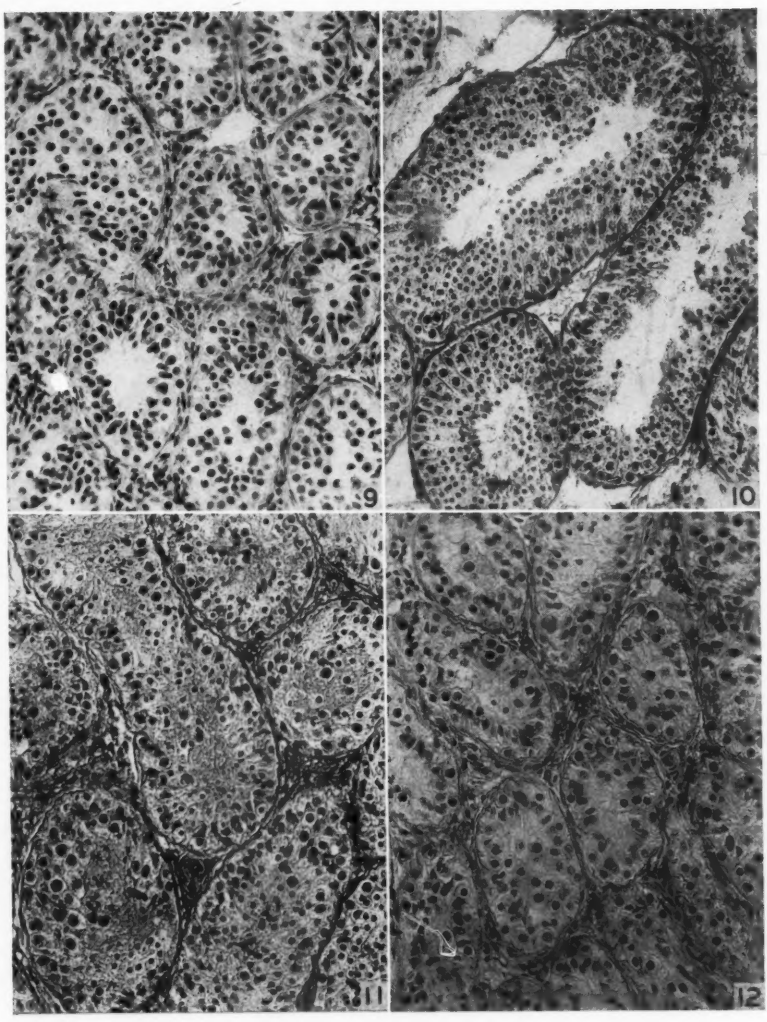


Fig. 9. Testis of *monkey* 597 (see fig. 5–8). Biopsy taken after 30-day treatment with 30 C.-S.U. of equine gonadotropin per day. Fig. 10. Testis of *monkey* 670. Biopsy taken at time of hypophysectomy. Treatment with equine gonadotropin was begun immediately after the ablation of the hypophysis.

Fig. 11. Testis of *monkey* 670. Biopsy taken after 30-day treatment with 250 C.-S.U. of equine gonadotropin per day. Fig. 12. Testis of *monkey* 670. Biopsy taken after 57-day treatment with 250 C.-S.U. of equine gonadotropin per day.

Immediate treatments. In 3 monkeys treatment was begun at the time of hypophysectomy (*monkeys* 377, 379, 670). In one of these (*monkey* 377) receiving 60 C.-S.U. daily, maintenance was incomplete for the 20-day period of treatment, some involution taking place during this period. In the other monkeys (*monkeys* 379, 660, receiving 100 and 250 C.-S.U. per day, respectively) there was complete maintenance for

18 and 20 days. Treatment of of one of these two (*monkey* 670) was continued for a total of 57 days. A biopsy taken on *day* 30 showed a definite regression (fig. 11) and one taken on *day* 57 when treatment was discontinued showed a furture regression, no sperm or spermatids being present although there were some spermatocytes (fig. 12). The testes were less firm than normal although in monkeys they do not become as flabby as in untreated hypophysectomized rats.

Measurements through the scrotum were made of the testes of *monkey* 670 at the time the biopsies were taken. The measurements were: at hypophysectomy, 45×32 mm.; at 10 days of treatment, 41×33 mm.; at 20 days, 41×31 mm.; at 30 days, 39×28 mm.; and at 57 days, 32×22 mm. Although subject to considerable error, these measurements are, nevertheless, sufficiently accurate to show a pronounced decrease in size although not as rapid a one as would have occurred had treatment not been given.

In this prolonged treatment the sex skin color which is characteristic of the sexually active adult male gradually receded after the 20th day and was entirely lost by the 57th day. A similar regression in sex skin color in females treated with pregnant-mare-serum was reported by Meyer and Gustus (11).

Dosage and Antibody Formation

Two reasons come to mind to explain the relative ineffectiveness of the treatments, namely, dosage and antibody formation.

Dosage. The dosages of gonadotropin used in these experiments ranged from 200 Danish mouse units (M.U.) for Antex or approximately 40 Cole-Saunders units (80 I.U.) to 300 C.-S.U. (600 I.U.) per day. In the animals receiving postponed treatments the effects from the lowest dosage used were definitely inferior to those from the higher dosages. In those receiving the immediate treatments, the 300 M.U. (60 C.-S.U.) dosage used on *monkey* 377 did not give as good a maintenance as did the larger dosages (*monkeys* 379, 670, receiving respectively 100 and 250 C.-S.U. per day). Although it is impossible to quantitate accurately the restorative or maintenance effects because of the number of animals used, nevertheless, the evidence indicates that maximum effects were secured with a dosage of between 100 and 200 C.-S.U. per day. An insufficient dosage, therefore, seems not to be the factor responsible for the failure to secure a satisfactory response when 100 or more units per day was injected.

The response in females also supplies evidence, although indirect, that the failure to secure a satisfactory testis response was not due to too low a dosage. In several hypophysectomized females it has been found (unpublished data) that a dosage of 25 C.-S.U. a day for 10 days gives a pronounced growth of the ovarian follicles. When 50 C.-S.U. was given daily for the last 3 days of the 10-day treatment, the follicles reached normal ovulating size. In some of the experiments on the males reported the total dosage over a 10-day period exceeded by 9 to 10 times that giving an excellent follicular growth.

Antibody formation. Assays of serum from monkeys treated with equine gonadotropin showed the presence of large amounts of antigonadotropic substance. No tests, unfortunately, were made earlier than 20 days during a period of treatment, but at that time (*monkey* 598), 0.6 cc. of serum partly repressed a dosage of 5 C.-S.U. of gonadotropin. A high titre of antigonadotropin was shown on *day* 25 and *day* 32 (*monkeys* 660, 597). When gonadotropin was given for only 10 days, assays made after a non-treatment period, showed as long as 205 days later a complete inactivation of 5 C.-S.U. of gonadotropin with 0.3 cc. of serum (*monkey* 635). Other animals tested

TABLE 2. ANTI-GONADOTROPIC ACTIVITY OF SERA OF MONKEYS TREATED WITH PREGNANT-MARE-SERUM GONADOTROPIN

Each total dosage of gonadotropin and of serum was injected into the test mice in 3 divided dosages an injection of each being given separately and subcutaneously each day for 3 days. They were autopsied 96 hours after the first injection. The mice were the Swiss strain and were 20–23 days old when the injections were begun. The dosage of gonadotropin is given in Cole-Saunders units (C.-S.U.)

No. Days Monkey Treated	No. of Test Mice	Dosage		Ovaries, mg.	Vagina	Neutralization[1]
		PMS, C.-S.U.	Serum, cc.			
Monkey 597♂[3], 200 C.-S.U./day beginning 60 days after hypophysectomy						
32	5	5	1.0	1.9(1.6– 2.4)	Closed	++++
	6	5	0.0	10.7(6.5–12.3)	Open	
	4	0	0.0	1.9(1.4– 2.7)	Closed	
40(8)[2]	4	5	0.5	2.8(2.6– 3.1)	2 Open, 2 closed	+++
	4	5	0.3	2.9(2.3– 3.4)	2 Open, 2 closed	++
	2	5	0.0	7.0, 10.0	Open	
Monkey 660♂[3], 300 .C-S.U./day beginning 20 days after hypophysectomy						
25[2]	3	5	1.0	3.5(3.3– 3.8)	Open	++
	6	5	0.5	4.4(2.7– 5.7)	Open	+
	8	5	0.0	11.6(8.6–14.1)	Open	
	4	0	0.0	3.4(2.2– 4.3)	Swollen	
Monkey 598♂[3], 400 C.-S.U./day beginning 61 days after hypophysectomy						
20	4	5	0.6	4.8(4.1– 5.5)	Open	++
	8	5	0.0	11.8(8.5–14.2)	Open	
	4	0	0.0	3.1(2.8– 3.3)	Closed	
20(106)[4]	4	5	1.2	3.2(2.6– 3.8)	Open	+++
	4	5	0.6	4.0(3.2– 4.8)	Open	+
	8	5	0.0	7.1(4.4– 7.2)	Open	
	5	0	0.0	3.7(3.0– 4.0)	Closed	
20(162)	3	5	1.2	3.0(2.6– 3.5)	Open	+++
	3	5	0.6	4.4(3.0– 5.6)	Open	+
	4	5	0.0	6.5(5.3– 7.6)	Open	
	4	0	0.0	2.2(2.1– 2.5)	Closed	
Monkey 577♀, 200 C.-S.U./day beginning 10 days after hypophysectomy						
10(15)[5]	4	5	0.6	2.5(2.3– 2.9)	1 Open, 3 closed	+++
	4	5	0.0	6.0(5.2– 7.8)	Open	
	4	0	0.0	2.4(2.1– 2.6)	Closed	
10(30)[5]	4	5	0.6	4.1(3.8– 4.4)	Open	++
	4	5	0.0	6.7(5.7– 9.0)	Open	
	5	0	0.0	2.6(2.0– 3.1)	Closed	
Monkey 635♀, 25 C.-S.U./day for 7 days and 50 C.-S.U./day for 3 days, beginning 10 days after hypophysectomy						
10(205)[6]	4	5	1.8	2.8(2.6– 3.3)	Closed	++++
	4	5	0.9	2.8(2.7– 2.9)	Closed	++++
	4	5	0.3	2.7(2.5– 2.8)	Closed	++++
	4	5	0.15	3.1(2.5– 4.3)	1 Open, 3 closed	++
	8	5	0.0	8.7(6.0–12.3)	Open	
	6	0	0.0	2.7(2.2– 3.4)	Closed	

TABLE 2.—*Continued*

No. Days Monkey Treated	No. of Test Mice	Dosage		Ovaries, mg.	Vagina	Neutralization[1]
		PMS, C.-S.U.	Serum, cc.			
Monkey 652 ♀, 25 C.-S.U./day for 8 days and 50 C.-S.U./day for 2 days beginning 10 days after hypophysectomy						
10(179)[7]	4	5	0.6	3.2(2.7– 3.9)	Closed	++++
	4	5	0.3	3.6(3.0– 4.5)	1 Open, 3 closed	+++
	6	5	0.0	6.0(5.1– 6.8)	Open	
Normal untreated monkeys, 579 ♀ and 609 ♀						
0	4	5	1.2	6.1(4.6– 7.6)	Open	None
	4	5	1.2	7.2(4.8– 8.1)	Open	None

[1] The degree of neutralization is indicated as follows: ++++ complete, +++ nearly complete, ++ very definite, + very slight but unmistakable. Judgment as to the degree of neutralization depended not only upon ovarian weights but also on ovarian structure and the uterine weights which are not given in the table.

[2] The blood for serum was taken 8 days after the termination of treatment.

[3] The mice employed in this test were 23 days old (at the maximum age) and were unusually large.

[4] The blood for this and for the next test was taken 106 and 162 days, respectively, after the termination of the gonadotropin treatment. These tests were not very satisfactory for a new batch of hormone against which the sera was tested did not have the potency of earlier batches. Nevertheless, a definite inhibition was shown.

[5] Blood for the serum tests was taken 15 and 30 days after the completion of the gonadotropin injections.

[6] Blood for serum was taken at autopsy 205 days after the termination of the gonadotropin injections. During this approximately 6-month period, desoxycorticosterone had been given for 100 days, 25 mg./day. No other treatment.

[7] Blood for serum was taken at autopsy 179 days after the termination of the gonadotropin injections. No treatment during this period except the injection for the last 12 days of 0.1 mg./day of estradiol benzoate.

5 to 6 months after the termination of treatment showed the presence of large amounts of antigonadotropic substance (table 2). Tests revealed no antigonadotropin in the serum of normal untreated monkeys.

DISCUSSION AND CONCLUSIONS

Eight sexually mature hypophysectomized rhesus monkeys have been treated with pregnant-mare-serum gonadotropin. In three of the animals, treatment was begun immediately after the ablation of the hypophysis; in two after a partial involution of the testes had taken place following hypophysectomy; and in three after complete involution. The dosages used varied from 40 to 300 Cole-Saunders units per day and the duration of the treatments extended from 9 to 57 days. In the animals receiving longer treatments biopsies of the testes were taken at intervals.

In the animals in which involution of the seminiferous tubules was complete or nearly complete, 100 or more C.-S.U per day, gave some repair. This repair was evidenced by mitoses in the spermatogonia and the appearance of a few spermatocytes. The repair did not extend to the formation of spermatids or sperm even with a dosage of 200 C.-S.U a day for 40 days. After 20 days there appeared to be no further improvement with a continuation of treatment, and apparently some regression even, took place.

In the experiments in which treatment was instituted immediately after hypophysectomy, maintenance seemed to be complete for 20 days but beyond this time involution became evident and in the one animal treated for 57 days the atrophy was pronounced, no sperm or spermatids being present at the termination of the injections. The gonadotropin, therefore, postponed but did not ultimately prevent involution during the two-month period of treatment.

The maintenance and restorative effects of the pregnant-mare-serum gonadotropin used are, therefore, very disappointing and are in contrast to the pronounced gonadotropic effects reported in hypophysectomized rats. The maintenance and restorative effects in monkeys are in fact not equal to those given by large dosages of androgen (unpublished data).

It has been pointed out above that the disappointing results are probably not due to an insufficient dosage, for much smaller amounts than were employed in the males induced in short periods (10 days) of treatment a pronounced response of the ovaries of hypophysectomized monkeys. This conclusion is further supported by the fact that for a brief period (20 days) there was complete maintenance in the animals receiving immediate treatment, and definite though not pronounced restoration in those receiving postponed treatment.

The shortness of the period during which the gonadotropin was effective appears to be due to its antigenicity. The antigonadotropin titre of the serum became high within 20 days and persisted for several months. One cubic centimeter or even less of serum inactivated 5 C.-S.U. of gonadotropin in some of the animals. It is evident that the dosages given would be inactivated by the circulating antibody.

That pregnant-mare-serum gonadotropin is antigenic has been shown by other investigators in a number of species of animals. Meyer and Gustus (10) demonstrated this in a series of treatments in monkeys. They found that with a dosage of 5 R.U. of purified extract the sex skin color increased up to 13 to 20 days and then regressed. Accompanying this regression was a decrease in the size of the ovaries. Inhibitory substance was present in the serum 67 days after the last injection. Meyer and Wolfe (11) with intermittent injections of crude and of purified pregnant-mare-serum gonadotropin in monkeys, found antigonadotropic substance in the serum of the treated animals but tests showed that the antigonadotropin decreased after 30 days and disappeared by day 59. Rowlands (12) and Hamburger (3) in rabbits, Thompson and Cushing (15) in dogs, and Rowlands and Spence (13) and Jailer and Leathem (4) in man, found that equine gonadotropin caused inhibitory substance to appear in the sera.

The findings on the antigenicity of these gonadotropin extracts reported in this paper are thus in accord with the observations of others and would appear to explain adequately, in monkeys, the failure of the extracts to maintain spermatogenesis, except for a brief period, and also to explain the failure to restore spermatogenesis in the hypophysectomized animals receiving the postponed treatments.

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EFFECTS OF LACTOGENIC AND GONADOTROPIC HORMONES ON HYPOPHYSECTOMIZED PREGNANT RATS

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THE LACTOGENIC FACTOR of the pituitary gland not only maintains pregnancy in rats hypophysectomized after implantation of ova has occurred (1), but in the absence of the pituitary it causes ova to implant and to develop into normal fetuses which may be delivered (2). Scanty evidence was adduced (1) that a pituitary gonadotropin, predominantly follicle-stimulating by usual criteria, also may maintain pregnancy. Since the early studies, establishing the vital rôle of the corpus luteum or its secretion, progesterone, in the nidation of the ovum and maintenance of pregnancy (3, 4, 5), have been confirmed in numerous ways (6–10), it is believed that hypophyseal substances maintain pregnancy after hypophysectomy by preventing involution of the corpus luteum. A view which is gaining favor, on the basis of studies by various investigators, postulates that the lactogenic factor of the pituitary (11, 12), or a principle closely associated with it (13), is responsible for activation of the corpus luteum. Formation of the corpus, however, is still thought to be brought about by a luteinizing gonadotropin from the anterior hypophysis.

As pointed outpreviously (1, 2) the concept that gonadotropins do not stimulate functional activity of the corpus luteum seems premature. The data which follow lend factual support to this criticism.

MATERIAL AND METHODS

Forty rats ranging in weight from 170 to 240 gm. were hypophysectomized at various stages of pregnancy before and after implantation (table 1) and injected with various substances.[1] Of these, 24 were treated with hypophyseal synergist,[2] 10 with lactogenic hormone,[3] and 6 with pregnant mare serum. Six animals were used as untreated hypophysectomized pregnant controls, 14 served as normal pregnant controls, and 7 as normal non-pregnant controls.

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[1] All of the substances used in these experiments were supplied by Dr. Erwin Schwenk, Schering Corp., Bloomfield, N. J.

[2] Synergist was prepared from sheep pituitaries. It was said to be chiefly follicle-stimulating with traces of luteinizing principle and probably of thyrotropic factor. A unit was defined thus: "The amount of synergist preparation which together with 15.0 international units of chorionic hormone will give the effect of 1 Cartland-Nelson unit in rats, i.e., an ovary of 55 mgm. weight." The following communication was received about its lactogenic potency: "Twenty milligrams injected into immature pigeons (according to Riddle) did not show any effect upon the crop glands. This would indicate that if prolactin were present it would be in a concentration of less than 2%."

[3] The potency of lactogenic hormone was stated to be 12.5 I.U. per mg. No information was supplied about its possible gonadotropic content. Results of preliminary experiments carried out by the writer on immature and adult hypophysectomized male and female rats indicate that the lactogen was feebly gonadotropic: (*a*), it caused late vaginal canalization in immature hypophysectomized female rats, with no visible microscopic effect on the ovaries; (*b*), it caused slight tubular development in hypophysectomized male rats; (*c*), it stimulated interstitial cell activity to a hardly perceptible degree.

TABLE 1. EFFECTS OF HYPOPHYSEAL AND CHORIONIC GONADOTROPINS, AND OF LACTOGENIC HORMONE IN HYPOPHYSECTOMIZED PREGNANT RATS

Group	Rat No.	Hypophysectomized, Days After Mating	Treatment: Daily dose in 1 cc. of solvent	Treatment: Number of days continued	First Blood in Vagina, Days After Mating	Pregnancy Maintained, Days After Mating	Number of Young in Litter	Autopsy Data: Killed, days after mating	Autopsy Data: Change from preoperative body weight (gm.)	Autopsy Data: Ovaries (mg.)	Autopsy Data: Adrenals (mg.)
Rats treated with synergist[2]											
I	81	2	5 U	20				20	−52	129	17
	82	3	20 U	9				9	−21	55	41
	92	2	10 U	12				12	−27	66	25
	93	2	10 U	12				12	−26	84	34
	94	1	10 U	22				23	−32	57	23
	98	3	15 U	21				22	−27	110	25
	99	2	15 U	20				21	−37	73	19
II	36	5	10 U	18	8	7		22	−36	94	35
	64	3	10 U	10	8	7		11	−24	81	43
	68	7	20 U	9	10	9		10	−22	92	43
	70	6	5 U	8	9	8		9	−29	104	48
	72	5	10 U	9	9	8		10	−25	106	36
	73	4	10 U	8	8	7		9	−23	99	35
	74	3	20 U	7	7	6		8	−18	56	32
	76	3	20 U	10	9	8		11	−19	71	22
	78	2	20 U	10	8	7		10	−17	69	34
	86	2	20 U	8	9	8		9	−22	70	26
	96	4	15 U	8	9	8		9	−23	97	33
III	30	7	10 U	17		27+	10 (dead)	27	+35	405	30
	57	8	10 U	21	15	27+	10 (dead)	27	+48	242	24
	58	8	20 U	21	15	27+	6 (dead)	27	+44	205	21
	63	8	10 U	20	15	27+	8 (dead)	27	+28	242	34
	65	3	10 U	14	15	26	3 (2 dead)	26	− 5	123	21
	80	2	10 U	24	14	24	8	26	− 5	115	25
Rats treated with lactogenic hormone											
IV	83	3	2 mg.	24				25	−38	36	23
	91	2	2 mg.	20				21	− 6	27	18
V	60	9	4 mg.	20	12	11		21	−18	41	40
	75	9	½ mg.	24	11	10		24	+ 3	48	29
	79	2	2 mg.	28	19	18		28	−15	34	22
	84	2	2 mg.	23	15	14		24	−19	33	26
	87	4	½ mg.	13	10	9		13	−17	31	28
	89	1	2 mg.	21	14	13		22	−12	46	26
VI	77	2	4 mg.	27	14	28+	3 (2 dead)	28	+12	51	33
	39	3	3 mg.	23	12	25+ (died)	8 (dead)	25	+62	50	36
Rats treated with pregnant mare serum											
VII	56	8	5 I.U.	21	12	11		22	−32	368	26
	61	9	5 I.U.	20	16	15		21	−19	337	28
	62	8	10 I.U.	17	11	10		18	−22	255	41
	67	6	20 I.U.	11	10	9		13	−18	351	40
	69	7	20 I.U.	9	9	8		10	−15	65	42
	71	6	40 I.U.	8	8	7		9	−14	60	36
Untreated hypophysectomized pregnant control rats											
VIII	32	9			11	10		22	−42	42	37
	34	9			10	9		22	−58	47	21
	97	3						21	−40	33	24
	101	3						21	−32	20	23
	95	4			8	7		8	−17	32	25
	100	4			7	6		8	−21	37	30
Normal pregnant control rats (average of 8 animals)											
IX					13.1	22	6.7	22	+22.3	73.3	58
Normal control rats (average of 7 animals)											
X										59	58

Pregnancies were timed by the following method. Daily vaginal smears were examined at 10:00 A.M. for spermatozoa or copulation plugs, and the vagina was inspected grossly at 4:00 P.M. for the presence of copulation plugs. All matings occurred in the 18-hour interval between 4:00 P.M. and 10:00 A.M. When spermatozoa or plugs were discovered in the vagina, rats were considered to be pregnant 24 hours, even though insemination could have occurred no earlier than 18 hours previously. The stages of pregnancy given in column 3 of table 1 are, therefore, without exception actually later than precise data would have established.

Hypophysectomies were performed at various times between the first and ninth days of pregnancy. It is known (14) that, in the rat, removal of the pituitary before the sixth day of pregnancy will prevent nidation of ova, and between the sixth and eleventh days it will invariably cause resorption of embryos. Completeness of hypophysectomy was checked in doubtful cases by sections of the pituitary capsule. Otherwise, the criteria relied upon were an initial rapid loss in body weight, marked shrinkage of the adrenal glands, and absence of any grossly visible gland tissue in the sella.

Daily injections were begun immediately after ablation of the pituitary. Each dose was administered in 1 cc. of an aqueous solution of trisodium phosphate and adjusted to PH 7.9. The length of treatment and dosage were variable and are summarized in table 1. Body weights and vaginal smears were recorded daily. The appearance of blood in the vagina prior to the expected time of the placental sign invariably was indicative of the cessation of pregnancy. In such instances (table 1) termination of pregnancy was considered to have started one day before detection of blood. Although such a generalization may be inaccurate, it may be justified as the basis of its value for purposes of comparison.

Animals were killed with ether. Adrenals, ovaries and thyroids were weighed in the fresh state and fixed in Bouin's fluid. Mammary glands were stretched on paper for preparation of whole mounts. Gross observations were recorded on the condition of ovaries, uteri, vaginae, and mammary glands, such records proving useful for comparison with specimens studied microscopically. Tissues were embedded in paraffin sectioned at 6 to 10μ and stained with hematoxylin and eosin.

RESULTS

A. *Rats Treated with Hypophyseal Gonadotropin (Synergist)*

Twenty-four rats were hypophysectomized between the first and eighth days of pregnancy and treated with 5 to 20 units of synergist (table 1). These animals were subdivided into three groups (*I, II, III*). In *Group I* pregnancy was not maintained; in *Group II* it was maintained for a time; in *Group III* pregnancy continued to expected term, or beyond, with or without parturition.

Group I. All animals were hypophysectomized before *day* 6 of pregnancy, when implantation normally occurs. Either the synergist failed to bring about nidation of fertilized ova, or the matings had been sterile. Losses in body weight and decreases in adrenal and thyroid weights were comparable with those known to occur in untreated hypophysectomized rats (15). The ovaries approached or exceeded in weight those of normal pregnant rats (*Group IX*) or of normal control rats (*Group X*). Histologically, however, the ovaries of the treated rats were readily distinguishable from those of pregnant or normal controls by the presence of numerous corpora lutea atretica (corpora with trapped ova) (cf. fig. 1 with fig. 9, 10), and of follicles exhibiting various degrees of luteinization of granulosa and theca cells. Corpora

lutea composed of small closely packed cells (fig. 2) and normal follicles of different sizes were also seen. The uteri revealed signs of follicular stimulation only, and the vaginae were cornified. There was no evidence of stimulation of mammary glands.

Group II. The pituitary was removed from 11 animals between *days* 2 and 7 of pregnancy. It may be questioned whether the synergist postponed the resorption of embryos in animals hypophysectomized on *days* 5, 6 or 7 of pregnancy (*rats* 36, 68, 70, 72). There is no doubt that implantation occurred in *rats* 86 and 96, since distinct deciduae were seen in the uteri. Small swellings which resembled resorption sites were present in the uteri of 5 animals (64, 73, 74, 76, 78); but in the absence of a decidua capsularis in such instances, it cannot be stated with certainty that nidation had preceded vaginal bleeding. Microscopically, swellings consisted either of generalized decidual transformations in the endometrium, or of distinct decidual growths projecting into the uterine lumen. Two such deciduae (in *rats* 72 and 86) surrounded blastocysts (cf. fig. 11, 12 with fig. 14). In general, however, the uteri appeared to be in transition from a luteal to an estrous phase. Mitoses in the epithelium were not infrequent. The vagina, too, revealed histological evidence of change from luteal to follicular domination, as indicated by marked epithelial proliferation subjacent to mucified areas, or by cornification of the vaginal epithelium. The ovaries contained corpora lutea with shrunken cells, and corpora lutea atretica not unlike those described in the first group (fig. 1, 2). There were, in addition to medium-sized normal follicles, some which were hemorrhagic and others which showed partly luteinized granulosa and thecal cells. A follicle which appeared to be recently ruptured was seen in an ovary of *rat* 86. From the appearance of the ovaries, uteri and vaginae it would appear that the hypophyseal synergist had induced marked follicular stimulation, a condition incompatible with the continuation of pregnancy. All mammary glands were atrophied or undergoing involution. The weight and histological appearance of the adrenal glands and thyroids were not unlike those of untreated hypophysectomized control rats. Loss in body weight, in general, was typical of that resulting after hypophysectomy.

Group III. Four animals were hypophysectomized a day or two after the expected time of implantation, and two underwent removal of the pituitary 3 or 4 days before that stage of pregnancy. Daily injections of 10 or 20 units of synergist in these animals caused unimplanted ova to implant and develop into fetuses some of which were delivered alive, or permitted implanted ova to develop into postmature fetuses which were not delivered before autopsies were performed. Vaginal bleeding characteristic of the placental sign occurred on *day* 14 or 15 of pregnancy in all but one of the animals. No placental sign was detected in *rat* 30; perhaps the bleeding was very transient. The first four animals listed in *Group III* (table 1) had not delivered by *day* 27 of pregnancy, at which time they were killed because of impending resorption, as indicated by vaginal bleeding. In all instances an initial loss in body weight was sustained following hypophysectomy. A week or 10 days later, however, the body weight had reached the pre-operative level, and thereafter weight increments were equal to those occurring in normal pregnant control rats. If delivery did not take place by *day* 25 or 26 *post coitum*, a sharp drop in body weight was noticed. As noted in the table the fetuses *in utero* were dead and, together with the placentae, were undergoing resorption. Development apparently continued for some days beyond the time of normal parturition, since the fetuses averaged considerably more in weight than term fetuses. The maternal loss in body weight was of such magnitude as to preclude an explanation on the basis of decreased weight of fetuses. In all probability the losses reflected weight changes in maternal tissues; and according to the work of

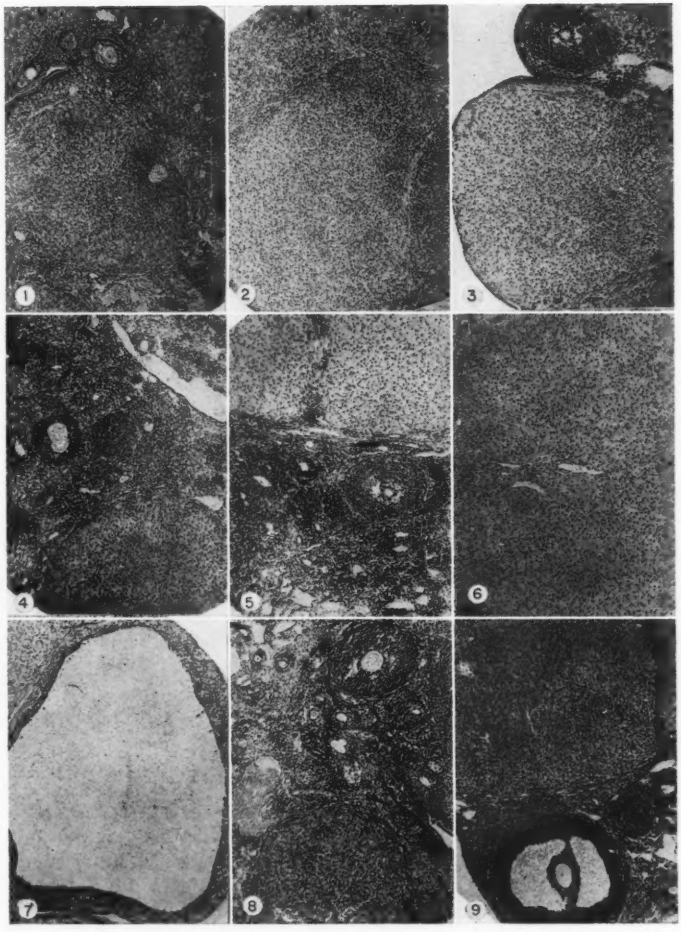

Fig. 1. SECTION OF OVARY typical of hypophysectomized rats treated with hypophyseal synergist and in which pregnancy was not maintained. Tissue from *rat* 72 (*Group II*) shows a corpus luteum atreticum (fragmented ovum seen at right margin of corpus). Fig. 2. DETAILS OF NON-FUNCTIONAL CORPUS LUTEUM in ovary shown in figure 1. Cells are shrunken and closely packed. Fig. 3. FUNCTIONAL CORPUS LUTEUM AND A SMALL FOLLICLE from hypophysectomized *rat* 80 (*Group III*) in which pregnancy was maintained by synergist. Cells of the corpus resemble those from a normal pregnant control (fig. 9). The follicle approaches in size and development one seen in a normal control rat (fig. 10).

Fig. 4. OVARY OF *rat* 84 (*Group V*). Lactogen had only a temporary stimulating effect. Segment of corpus luteum (lower right), atretic follicle (center, left), and generalized interstitial atrophy give an appearance not unlike that in an untreated hypophysectomized pregnant control (*rat* 95, *Group VIII*, fig. 8). Fig. 5. OVARY FROM HYPOPHYSECTOMIZED *rat* 77 in which pregnancy was maintained by lactogenic hormone. The corpus luteum is stimulated, but the remainder of the gland is atrophic. Fig. 6. LARGER PORTION OF THE CORPUS LUTEUM appearing in the upper left hand corner of figure 5. Compare with figure 9.

Fig. 7. CHARACTERISTIC CYSTIC DEVELOPMENT IN OVARY OF hypophysectomized pregnant *rat* 56 (*Group VII*) following injections of PMS. Fig. 8. OVARY OF *rat* 95 (*Group VIII*), an untreated hypophysectomized pregnant control. Fig. 9. OVARY FROM NORMAL PREGNANT control rat at term.

All figures magnified 53×.

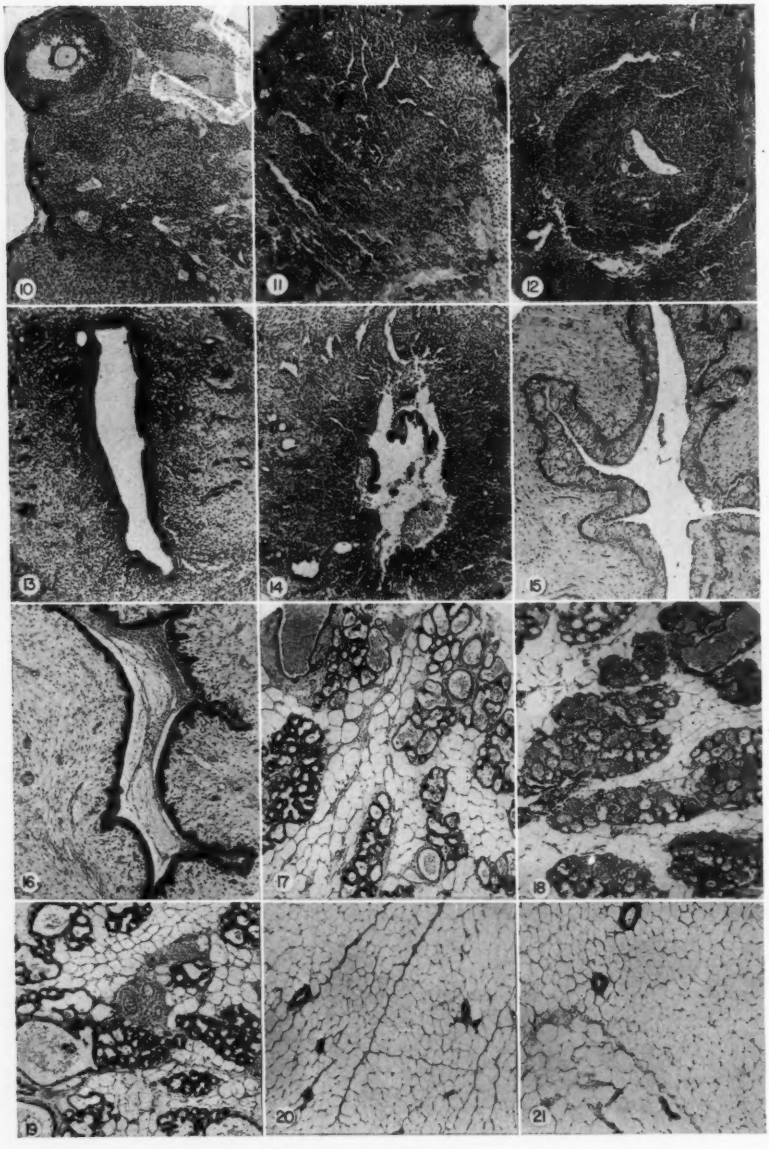

Fig. 10. Ovary from non-pregnant normal control. Fig. 11. Atrophying decidua capsularis surrounding degenerating blastocyst (small dark mass in center) from *rat 72* (*Group II*). Stalk of decidua is above, with uterine lumen to left and right of it. Fig. 12. Degenerating blastocyst (*center*) and decidua capsularis embedded in the uterine wall of *rat 86* (*Group II*). Hemorrhage may be seen in and around decidua.

Fig. 13. Uterus of *rat 84* (*Group V*). Retrogression from a previous luteal phase. Fig. 14. Uterus of *rat 95* (*Group VIII*), an untreated hypophysectomized control. Observe hemorrhage and desqua-

Newton and Beck (16), such changes are precipitated by involution of the placentae. *Rat 65* gave birth on the 26th day to 3 fetuses, 2 of which were stillborn. Eight living young were delivered by *rat 80* on *day 24 post coitum*. It should be noted that injections in *rat 65* were discontinued two days after the placental sign, with no deleterious effects on the subsequent course of pregnancy and parturition. After parturition both animals (*65* and *80*) weighed 5 gm. less than pre-operatively, while normal pregnant animals (*Group IX*) showed a gain in weight.

Ovaries of all animals were large and revealed swollen corpora lutea characteristic of pregnancy, together with a few small or medium sized follicles (fig. 3). Mucification typical of pregnancy was seen in the vaginae (cf. fig. 15 and 16). Without exception the mammary glands were highly stimulated, and the alveoli and ducts contained a milky fluid (cf. fig. 17 with fig. 19, 21). Of special interest is the fact that *rat 80* produced enough milk to sustain her litter of 8 for 3 days. The stomachs of the young were milk-distended as late as 48 hours after delivery. No stimulation of adrenals or thyroids was noticed.

B. *Rats Treated with Lactogenic Hormone*

Rats in this series were hypophysectomized 1 to 9 days after mating and injected daily with 0.5 to 4 mg. of lactogenic hormone. As in the synergist series, rats injected with lactogen fell into 3 groups (*IV*, *V*, *VI*, table 1). There was no maintenance of pregnancy in *Group IV*, partial maintenance in *Group V*, and prolonged gestation in *Group VI*.

Group IV. Two females, hypophysectomized at pre-implantation stages of pregnancy and injected with lactogen, were in no way affected by the hormone. So far as could be ascertained, losses in body and organ weights and microscopic changes in tissues were identical with those which might be expected in hypophysectomized control animals (fig. 8). It is possible that matings in these animals had been sterile.

Group V. Lactogenic hormone in daily doses of 0.5 to 4 mg. was administered to 6 females hypophysectomized 1 to 9 days *post coitum*. At autopsy resorption sites were found in the uteri of 4 animals; they were absent in *rats 60* and *84*. Resorption sites in the uteri of 3 animals hypophysectomized on *day 1*, 2 or 4 of pregnancy indicated that lactogen had induced nidation. All rats, except *75* which was hypophysectomized relatively late, underwent steady reductions in body weight. It may be noted that blood appeared in the vaginae of 3 rats hypophysectomized before implantation (*79*, *84* and *89*) on or after the time of the normal placental sign. The reason for this is not clear, particularly since resorption sites in these animals were not large, and since bleeding was not profuse or of long duration. It is not unlikely that implantation, and hence post-implantation abortion, was delayed. This idea is tempting in view of the fact that delayed implantation is a phenomenon of regular occurrence in impregnated rodents which are suckling young, and in which the pituitaries are presumably secreting large amounts of lactogenic hormone. In weight and histological

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mated epithelium in lumen. Fig. 15. MUCIFICATION IN VAGINA of hypophysectomized *rat 80* (*Group III*), in which pregnancy was maintained with synergist.

Fig. 16. VAGINA OF *rat 95* (*Group VIII*), an untreated hypophysectomized control. Degeneration of epithelium is well under way. Fig. 17. MAMMARY GLAND OF HYPOPHYSECTOMIZED *rat 80* (*Group III*), in which pregnancy was maintained with synergist. Fig. 18. MAMMARY GLAND OF HYPOPHYSECTOMIZED *rat 77* (*Group VI*) in which pregnancy was maintained with lactogen.

Fig. 19. MAMMARY GLAND of normal pregnant control rat at term. Fig. 20. MAMMARY GLAND OF HYPOPHYSECTOMIZED *rat 84* (*Group V*) in which pregnancy was not maintained with lactogen.

Fig. 21. MAMMARY GLAND of non-pregnant normal control.

All figures magnified 51×.

appearance, the ovaries in this group were not unlike those of hypophysectomized pregnant controls (cf. fig. 4, 8). In general the uteri (fig. 13) and vaginae exhibited evidence of retrogression from a condition built up under the influence of corpus luteum hormone. Except for slight stimulation in *rat 89*, all mammary glands were atrophic (fig. 20), as were adrenals and thyroids.

Group VI. Two rats hypophysectomized on *day 2* or 3 of pregnancy were injected daily with 4 or 3 mg. of lactogen. This treatment resulted in implantation and subsequent development of ova. Neither animal delivered; one was killed 28 days after mating (77), and one died after 25 days (39). One of three fetuses was found alive in 77, and 8 dead fetuses were recovered in 39. The fetuses were postmature. Both mothers had gained steadily, after an initial post-operative loss, but 77 began to lose weight a few days before autopsy. The ovaries revealed large corpora lutea indistinguishable from those of late normal pregnancies (cf. fig. 5, 6 with fig. 9). These were the only structures stimulated; the rest of each ovary, except for growth of small follicles, was atrophied (fig. 5). Mammary glands showed lobulo-alveolar development, and some fluid was present in the acini (fig. 18). Mucification of the vagina was typical of late pregnancy. There was no stimulation of adrenals or thyroids.

C. *Rats Treated with Pregnant Mare Serum*

Group VII. No animal was hypophysectomized before presumptive implantation. Daily doses of 5 to 40 I.U. of pregnant mare serum did not prevent prompt abortion, except possibly in *rat 61*. All animals lost weight. Ovaries of animals treated for relatively long periods were enormously enlarged, and exhibited many cystic and hemorrhagic bodies (fig. 7). Corpora lutea were variable in size and appearance. There was luteinization of granulosa in some follicles. Uteri and vaginae in most instances were estrous in appearance. Slight stimulation of mammary ducts was observed in all except *rat 69*. There was no stimulation of adrenals or thyroids.

DISCUSSION

The data of this report, together with those previously published (1, 2), demonstrate that successful treatment with a hypophyseal gonadotropin or with a lactogenic principle, was characterized by the following events in hypophysectomized pregnant rats.

a). If hypophysectomy preceded nidation of ova, implantation occurred. Subsequent development was similar to that of ova implanted prior to hypophysectomy: i.e., they developed into mature or postmature fetuses, with occasional deliveries as late as *day 26* of pregnancy.

b). Animals in which pregnancy was maintained underwent full mammary development with production of milk. Exhaustion of the supply of milk by suckling young was not prevented by postpartum continuation of treatment.

c). When pregnancy was maintained, the mother, after an initial post-operative loss, gained in body weight as did normal pregnant rats. After delivery or after fetuses had died *in utero*, there was a precipitate decrease in body weight.

The indispensability of the corpus luteum for implantation of ova and maintenance of at least early stages of pregnancy has been well established (3, 4, 5). There is no reason to doubt that hypophyseal synergist and lactogenic hormone maintained pregnancy through an ability to stimulate corpora lutea to secrete progesterone. Since the synergist used in the present study was a gonadotropin probably free of all but traces of lactogenic principle,[2] the concept that gonadotropic substances may only provoke formation of corpora lutea without initiating function (11, 12, 13) is contra-

dicted. Evidence that gonadotropins may have at least a brief corpus luteum-stimulating effect is found also in the studies of Robson (17, 18).

Our incomplete understanding of the factors concerned in the termination of pregnancy is emphasized in the reviews by Snyder (19) and Newton (20). Under the influence of synergist or lactogen, delivery occurred after normal term, or fetuses were retained as late as *day 28* of pregnancy, when autopsies were performed. The most likely explanation would seem to be that the hormone administration resulted in prolonged functioning of the corpora lutea. Such a view is in keeping with the conclusions of Teel (21) and of Snyder (22). It does not seem probable that uterine distension (23) played any important rôle in determining the duration of pregnancy in these experiments.

Milk production is difficult to explain in the hypophysectomized rats in which pregnancy was maintained. Studies by Newton and Lits (24) and Newton and Richardson (25) suggest that the placenta was probably concerned in evoking such mammary activity. Transient lactation *post partum* has been observed in rats (26) and mice (25) hypophysectomized in the latter half of pregnancy. Noteworthy in the present study is the copious lactation, lasting at least 48 hours, in *rat 80* which had been treated with synergist.

A footnote[2] explains that in conventional terms the hypophyseal synergist used in this investigation is predominantly follicle-stimulating. This substance has been tested in this laboratory on immature and adolescent hypophysectomized male rats, and doses similar to those employed in the hypophysectomized pregnant females were found to be chiefly gametokinetic. In the hypophysectomized pregnant females this same substance induced previously formed corpora lutea to secrete progesterone; it caused formation of new corpora, which might or might not have been functional; it produced corpora lutea atretica, possible rupture and luteinization of at least one follicle, hemorrhagic follicles, and medium-sized follicles. Such findings re-emphasize the view (27–30) that theories ascribing various gonadal reactions to a multiplicity of gonadotropic hormones (31, 32) are not above criticism.

SUMMARY AND CONCLUSIONS

Rats were hypophysectomized 1 to 9 days after mating and injected with various doses of a hypophyseal gonadotropin (synergist), lactogenic hormone, or pregnant mare serum. Administration of synergist or lactogen, but not of mare serum hormone, prevented interruption of pregnancy in a number of animals hypophysectomized before or after the expected time of implantation of ova. Treatment maintained pregnancy for varying periods of time. In some animals resorption of embryos took place within a few days after treatment was begun; in other animals pregnancy was terminated by delivery of postmature fetuses. Fetuses were retained as late as 28 days *post coitum*.

After an initial post-operative lag, animals which delivered, or which retained fetuses, gained in body weight as did normal pregnant rats, They suffered rapid losses in body weight after delivery or after postmature dead fetuses and placentae began to undergo resorption.

The mammary glands of all treated rats in which fetal development occurred were highly stimulated, and the alveoli were distended with secretion. One female which delivered 8 young lactated for at least 48 hours, the litter dying on the third day.

The results are discussed, with special emphasis upon two points: *a*), gonadotropic substances may not only cause the formation of corpora lutea, but may also

stimulate them to secrete progesterone; b), criticism of theories postulating a multiplicity of gonadotropic hormones.

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A QUANTITATIVE STUDY OF THE PARAVENTRICULAR NUCLEUS AND ITS ALTERATION IN HYPOPHYSECTOMY

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FROM THE EXTENSIVE retrograde change observed in the supraoptic nucleus after hypophysial stalk resection (1, 2, 3) it appears that practically all of the fibers from the supraoptic nucleus enter the neurohypophysis. While some cells of this nucleus remain intact after the usual hypophysectomy, these may be assumed to be connected with the median eminence of the tuber cinereum which is left intact and which is now regarded as part of the neurohypophysis (2–5).

The relation of the paraventricular (filiform) nucleus to the innervation of the hypophysis is not so evident. Numerous authors have reported varying degrees of cell loss in this nucleus of various so-called hypophysectomized animals. Magoun and Ranson (3) in a study of 11 monkeys estimated that about 20 per cent of these atrophy as a result of severing the infundibular stem. Rasmussen (1) was not so sure that it occurred regularly in the rat. In the dog he was less certain of the reaction of the nucleus to stalk resection. In the human being (6), there is apparently some loss, but due to the great variability in the prominence of the nucleus in man the actual degree of response is not certain. In lesions of the brain which destroy the regions about the supraoptic nucleus, the large cells of the paraventricular nucleus of the coressponding side largely disappear (7). With more complete destruction of the median eminence of the tuber cinereum, White and Heinbecker (8) have shown in dogs that there may be almost a complete disappearance of the supraoptic nucleus, as well as a great loss of cells in the paraventricular nucleus.

The cell losses reported by the various investigators were determined, for the most part, by comparing serial sections of the hypothalamus of operated animals with similar sections from normal controls and noting any decrease in the cell density of the nucleus in question. No detailed quantitative studies have been made to determine the effects of hypophysectomy and stalk resection upon the paraventricular nucleus. Indeed, except for the work of Morgan, Vonderahe and Malone (9, 10) apparently no serious attempts at quantitative methods have been encountered in connection with this nucleus. The data reported here were gathered by making actual cell counts upon the paraventricular nucleus of both normal and hypophysectomized albino rats, in the hope that there would be obtained a more exact knowledge of the extent to which this nucleus is connected with the principal part (processus infundibuli) of the neurohypophysis.

MATERIAL AND METHODS

I am indebted to Dr. R. C. Truex and colleagues in the Department of Anatomy of Columbia University for furnishing the brains of a number of hypophysectomized rats; also to Dr. R. M. Reinecke of the Department of Physiology of the University of Minnesota, who supplied a number of normal and hypophysectomized animals.

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I am also deeply grateful to Dr. R. L. Merrick in the Department of Anatomy of the University of Minnesota for allowing me to use several serially sectioned rat brains, which have been used in studies on the supraoptic nucleus. All of the treated animals had been hypophysectomized by transpharyngeal suction.

A total of 26 animals were used. Fourteen adult white rats served as controls, ranging in age from 356 to 497 days. Merrick (11) has found that age is not critical for the number of cells in the supraoptic nucleus. On the assumption that the same thing is true of the paraventricular nucleus, there is probably no serious error introduced by not having littermate controls in all cases.

Of the 12 rats operated upon when from 21 to 149 days of age, 9 were found to have no processus infundibuli, while in the other 3 rats portions of the processus infundibuli or posterior lobe proper, were found to be still present. All of the treated animals had an intact median eminence. Some of the hypophysectomized animals had sufficient anterior lobe (pars distalis) remaining to continue growth to various degrees. Table 1 gives further details on the individual animals.

All of the treated animals were allowed to live from 30 to 241 days post-operatively. In all but 3 animals the time allowed to elapse after hypophysectomy was over 100 days. The three exceptions were 30, 32 and 41 days respectively. This long period of time was allowed to elapse so that the degenerating cells would have time to disappear completely, thus avoiding the necessity of having to decide upon the number of cells affected on the basis of chromatolysis. The typical large cells of the paraventricular nucleus normally contain a perinuclear region which is free from prominent Nissl granules, so that chromatolysis is not so readily determined.

All specimens were fixed in 10 per cent formalin, cleared in dioxan, embedded in paraffin and sectioned serially at 10μ in a frontal plane. Some of the animals had also been perfused with 10 per cent neutral formalin. Every 5th or every 6th section in the ribbon was mounted. Cresyl violet was used for staining in all cases.

In determining the number of cells present, every cell with a distinct nucleolus was counted. However, after a preliminary examination of the nucleus it was deemed feasible to count only those cells which had the typical morphology characteristic of the cells of the supraoptic nucleus (i.e. cells with peripheral arrangement of the Nissl substance, eccentric nucleus with a large prominent nucleolus, and a perinuclear area free of coarse Nissl granules). This will be further explained in connection with the statement of the results.

The high dry objective of a binocular microscope with a mechanical stage was used in counting. With such a magnification, the morphology of the individual cells could be carefully examined and only the proper cells included. As an aid in counting, a Howard ocular micrometer disc was used in one of the eye-pieces of the microscope. This ocular micrometer squared the field and subdivided it into 36 smaller squares. The mechanical stage permitted careful exploration of the entire nucleus. The procedure followed was very similar to that employed in making blood cell counts, with reference to cells whose nucleoli fell within or touched the boundaries of the squares.

After the total number of cells (of the particular type just mentioned) in each nucleus had been determined, the sum was multiplied by 5 or 6, depending upon whether every 5th or 6th section in ribbon had been mounted. It has been shown that in a section of the thickness used no appreciable error is introduced due to split nucleoli, if only distinct nucleoli are counted (12).

RESULTS

Observations of normal animals. Rostrally the paraventricular nucleus begins with the appearance of a few large dark cells, which are scattered between numerous

small light staining cells. In sections taken more posteriorly (caudally) these cells expand more or less abruptly into a mass with a triangular configuration, as shown in figure 2. While the dorsal extremity of this mass is expanded laterally, the longest side of the triangle is the base, which lies parallel to the wall of the ventricle. Continuing posteriorly, after having passed the point with the greatest number of cells in a frontal section, the ventral portion of the nucleus is occupied by small light staining cells of the periventricular gray matter. The dorsal expanded portion persists however, and extends further laterally, so that the longest side of the triangle becomes perpendicular to the wall of the third ventricle instead of parallel. The cells in this portion of the nucleus then also fade out rapidly. The anterior-posterior (cranial-caudal) length of the nuclear mass, or the point at which the first typical dark cells begin to appear anteriorly and disappear posteriorly, was found to be .65 to .70 millimeters. This length was found to be quite constant in the normal rats. In the hypophysectomized animals the length was more difficult to determine, but seemed to be slightly shorter where great cell loss was encountered.

In frontal sections of the rat hypothalamus taken through the center of the paraventricular nucleus and prepared with a Nissl stain, (fig. 2) the nuclear mass stands out quite prominently. Its boundaries are rather distinct and it contains many large and medium-sized dark staining cells with the characteristics of the cells of the supraoptic nucleus. The paraventricular nucleus is generally considered to be composed of two portions, a dorsal-lateral part called the magnocellular portion containing very large dark staining cells, and a ventromedial part or paravocellular portion, composed of medium-sized dark cells (13). Intermingled with these characteristic cells, however, are also found medium and small-sized lighter staining cells similar to the cells of the general hypothalamic periventricular system. As the paraventricular nucleus begins to fade out posteriorly, these light staining cells become relatively much more abundant and it becomes increasingly more difficult to determine the boundaries of the nuclear mass. Some of the larger lighter staining cells, which no doubt are a part of the periventricular gray matter, have a morphology which is more or less transitional between the medium-sized dark staining cells, and it was difficult in many cases to decide whether these cells should be included with the typical cells or omitted from the count.

Originally, it was thought that it might be feasible to establish certain definite boundaries for the paraventricular nucleus and to make separate counts on the typical dark cells and on the lighter staining cells. It was hoped that by this method the reaction of both types of cells to infundibular stalk resection could be determined. After much study it seemed impractical to count these small and medium-sized cells because they are not sharply delineated from the adjacent gray matter. In other words, the limits of the nucleus as far as the smaller cells are concerned are too indefinite.

The following criteria were used in selecting the appropriate cells to be included in the count: *a*), a peripheral arrangement of coarse Nissl granules; *b*), an eccentric nucleus with a large prominent nucleolus; and *c*), a perinuclear area free of Nissl substance.

In frontal section the maximum number of cells counted ranged from about 100 to 130 per side. The total cells on one side ranged from 2430 to 3714 with an arithmetic mean for the 14 controls of 3067. There was no significant difference between the right and left side.

Observations on operated animals. The paraventricular nucleus in the hypophysectomized animals retains its cross-sectional triangular shape, but the boundaries of the nuclear mass become more difficult to determine, especially in the anterior and posterior extremities. With a diminution in the number of the typical cells with

prominent peripherally located Nissl granules, the edges of the nucleus seem to blend more closely with the general periventricular gray matter (fig. 3).

The greatest loss of cells was encountered in the dorso-lateral or magno-cellular portion of the nucleus. Here the loss is very apparent even with casual observation. In the ventro-medial portion of the nucleus, however, the loss of cells is not so obvious.

In conventionally hypophysectomized animals in which the infundibular process is entirely gone, the range of the maximum number of cells in a single section was from about 50 to 80 in each nucleus. In animals in which a portion of the infundibular process was found to be still present, the maximum cell counts per section were intermediate between the values given for normal and for animals with complete removal of the processus infundibulis.

Although no actual quantitative work was done on the light staining cells found within the limits of the paraventricular nucleus, it seemed from inspection that these

TABLE 1. NUMBER OF CHARACTERISTIC CELLS IN THE PARAVENTRICULAR NUCLEUS OF HYPOPHYSECTOMIZED RATS AND OTHER DATA ON THE ANIMALS

Rat	Number of Cells		Age when Operated, days	Weight at Operation, gm.	Days Operated	Weight when Killed
	Right	Left				
1	2315	2685	62	180	241	*
2	1760	1955	73	200	230	
3	2685	2810	141	219	110	247*
4	1780	1662	139	188	41	213
5	2154	1806	116		162	
6	1910	2105	82	131	30	89
7	1780	2320	21	45	154	236
8	2310	2120	77	205	149	155
9	1940	1860	149	235	149	197
10	1840	1680	148	223	101	188
11	1902	2046	143	227	32	202*
12	2016	2232	140	211	130	

* Portions of processus infundibuli present.

cells persisted when the larger dark staining cells diminished in number. Evidently this nucleus is a mixture of cells functionally as well as morphologically. This is supported by the studies of Morgan, Vonderahe, and Malone (9, 10). From the recent work of Heinbecker and White (14) the caudal portion of the paraventricular nucleus appears to be related to fat storage.

In the 9 rats in which the infundibular process was completely removed, the total cells per nucleus (on one side) varied from 1662 to 2320. It should be noted that even this maximum value falls below 2430 which was the minimum number found in the controls. The mean value (1958) is 35 per cent less than the normal. Table 1 contains the detailed data on these animals. If the right and left nucleus are combined in each animal, the hypophysectomized animals are found to have a mean of 3916, which is 2000 below the mean of 6135 for the controls (see fig. 1, 'partial' and 'complete' hypophysectomy in this table refer only to processus infundibuli). According to Fisher's t test for significance of difference between two means of small samples, the possibility that such a difference is due to chance alone is very remote (t is 11.02, N is 21, and p is less than .01).

Since the cell loss encountered is brought about by a retrograde degeneration due to severing of nerve fibers, it may be assumed that of the neurons in the paraventricular nucleus at least 35 per cent of the cells send their axons through the infundibular

stalk into the processus infundibuli. It appears however, that there is some irregularity in the number of nerve fibers passing from the paraventricular nucleus into the posterior lobe of the hypophysis. In *rat 8* it was found that although the supraoptic nucleus showed the greatest cell loss found in any of the operated animals, the paraventricular nucleus showed only a moderate cell loss (table 1). The supraoptic nucleus

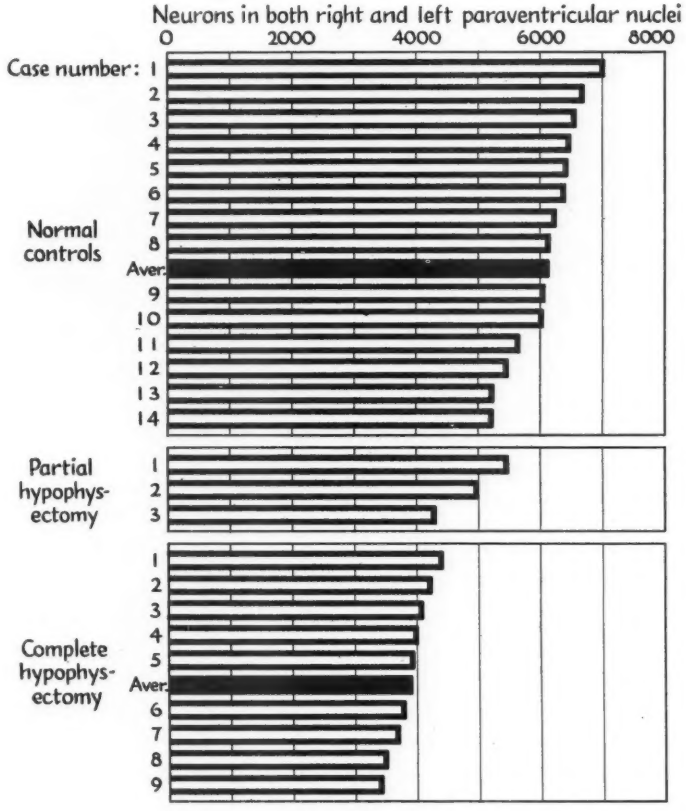

Fig. 1. Number of characteristic cells in paraventricular nuclei of normal rats, of rats ith incomplete removal of processus infundibuli ('partial hypophysectomy'), and of rats without processus infundibuli ('complete hypophysectomy').

had disappeared almost completely with only a few scattered cells still present. In other animals in which much lower counts were found in the paraventricular nucleus, a greater proportion of cells remained in the supraoptic nucleus. Judging from the reaction in the supraoptic nucleus this persistence of cells in the paraventricular nucleus cannot be explained on the basis that the point of interruption of the fibers was unusually low. Minor differences in the number of cells that disappear might be due solely to the fact that the point of interruption of the fibers differed in the various cases, leaving a variable amount of neurohypophysis still attached.

Observations on animals with incomplete removal of the infundibular process. It has been definitely shown that the loss of cells in the supraoptic nucleus after stalk resection is correlated with the height at which the stalk is cut. The greatest loss occurs when the median eminence of the tuber cinereum is involved (3). The data gathered on the 3 animals with portions of the infundibular process still present show that the cell loss in the paraventricular nucleus is also dependent upon the amount of neurohypophysis destroyed. This is graphically demonstrated in figure 1. The three rats in the 'partial hypophysectomy' group (*rats* 1, 3 and 11 of table 1) had an incomplete removal of the infundibular process. The counts on the incompletely operated animals

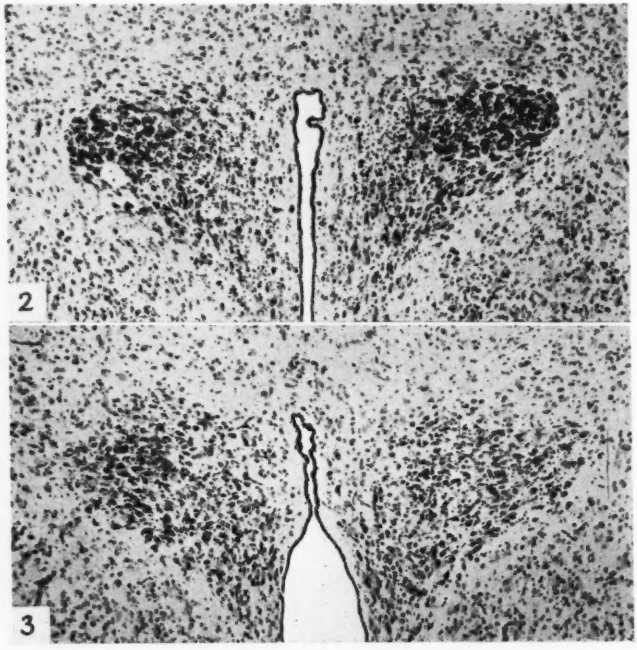

Fig. 2. PHOTOMICROGRAPH OF FRONTAL SECTION THROUGH DENSEST PORTION OF PARAVENTRICULAR NUCLEI OF NORMAL ADULT ALBINO RAT. 10μ Cresyl violet. Fig. 3. PHOTOMICROGRAPH OF FRONTAL SECTION THROUGH DENSEST PORTION OF PARAVENTRICULAR NUCLEUS OF ADULT HYPOPHYSECTOMIZED RAT IN which the processus infundibuli had been completely removed for 3 months. Same thickness and technique as in figure 2. Note the great loss of cells in the dorsolateral (magnocellular) portion.

are shown to lie intermediate between the controls above and the completely operated animals below. *Rat* 11 (table 1) showed the greatest cell loss of the 3 animals with posterior lobe tissue still attached, with 1902 and 2046 cells in the right and left nuclei respectively. In *rats* 1 and 3 (table 1) counts of from 2315 to 2810 were found. Further details on these animals are found in table 1.

Although the values mentioned above for the paraventricular nuclei in partial hypophysectomy (with reference to processus infundibuli) fall below the mean of the normal controls (3067), two of them are quite close. In all 3 rats however, a definite cell loss was noted in the supraoptic nucleus, and using cell loss in this

nucleus as a criterion, these animals may be definitely separated from the controls. Evidently they represent cases in which only a portion of the nerve fibers in the hypothalamic-hypophysial system were interrupted, and contribute further evidence that the number of cells remaining in the paraventribular nucleus as well as the supraoptic nucleus is dependent upon the amount of neurohypophysis remaining undisturbed and attached to the brain.

SUMMARY

Quantitative methods were used on the paraventricular nucleus of rats to determine the number of typical dark cells with prominent peripherally arranged Nissl granules in both normal controls and so-called hypophysectomized animals (with the median eminence of the tuber cinereum left intact, as is usually the case with transpharyngeal suction technique). Twenty-six albino rats were used for this study, of which 14 were controls. Nine had complete removal of the infundibular processes and 3 had some posterior lobe tissue intact. Frontal sections, 10μ thick, were stained with cresyl violet.

In the normal nucleus a range of from 2430 to 3714 characteristic cells was found, with a mean of 3067 for an individual nucleus or 6135 for combined right and left. In the completely operated animals counts of from 1662 to 2320 were obtained, with a mean of 1958 per side or 3916 for the two sides. This is a loss of about 35 per cent of the cells. The incompletely operated animals which still had posterior lobe tissue present showed intermediate counts. Since the cell loss appears to be caused by a retrograde degeneration following the severing of nerve fibers, it can be assumed that at least 35 per cent of the large characteristic neurons in the paraventricular nucleus send their axons through the infundibular stalk into the posterior lobe of the hypophysis.

The loss of typical dark staining cells was most prominent in the lateral wing or magnocellular portion. The light staining cells within the limits of the paraventricular nucleus apparently show no reaction to stalk resection.

Apparently there is some irregularity in the relative percentages of nerve fibers from the supraoptic and paraventricular nuclei that enter the hypothalamic-hypophysial system since the loss is not always parallel in the two, although some other factor, such as a difference in the degree of vascular interference, may be responsible for the variation.

The work was done under the direction of Dr. A. T. Rasmussen, whose assistance and encouragement are gratefully acknowledged.

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EFFECT OF EARLY HYPOPHYSECTOMY ON HYPOTHALAMIC OBESITY[1]

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THE HYPOTHALAMIC LESIONS which produce obesity in the rat have already been described in considerable detail by Hetherington and Ranson (1, 2). At the same time, although the question of finer cytological changes in the hypophysis was left open, the lack in most of the animals of any gross histological damage to the gland was noted. This observation did not eliminate the possibility that the hypophysis might play some mediating rôle between the hypothalamus and fat metabolism, and it was considered possible that the presence of the hypophysis, or at least of a portion of the anterior lobe might even be essential for the appearance of adiposity after the placing of hypothalamic lesions.

To be sure, in a few of the earlier workers' experiments some evidence was to be found that this hypothetical limitation might not be the case. For instance, among the rats operated by Smith and Graeser (3), Smith (4), and Crooke and Gilmour (5), who produced some cases of adiposity, several are mentioned of which it was said that 'no typically staining anterior lobe cells were found,' or 'no anterior lobe tissue was seen.' In at least some of these cases some neural lobe tissue was present. A number of workers (6–9) who used dogs have alluded to a few fat animals which retained no pituitary. Aschner (10, 11) even believed that the complete hypophysectomies he performed on puppies were responsible for their adiposity. Of course, he later (12) revised this opinion somewhat, and it seems safe to believe that he had injured the hypothalamus of his dogs; but at any rate it should not be overlooked that he probably observed adiposity in the *absence* of the hypophysis.

Inasmuch as the histological controls in some of these experiments did not seem to be above question, and since proof for the direct cause-and-effect relationships existing between certain specific hypothalamic lesions and so-called 'hypothalamic obesity' has only recently been put on a firm basis, it seemed desirable to examine once more, critically, the case for dispensability of the hypophysis in this syndrome. This has been done by superimposing upon each other two separate, simple, definitely controllable procedures: the one, hypophysectomy, and the other, the making of hypothalamic lesions by the Horsley-Clarke technic.

METHODS

Large bilateral lesions were placed by means of the Horsley-Clarke stereotaxic instrument in the hypothalamus of 10 rats weighing between 75 and 135 gm. by the technic already described (1). After 6 to 10 days had elapsed and all but one of the animals had largely recovered from the acute symptoms of the first operation, 9 of them were hypophysectomized via the ventral, parapharyngeal route.[2] The tenth

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rat (*Rd7*) displayed vestibular difficulty and was not hypophysectomized. All operated animals had normal litter-mate controls. The rats were weighed and their body lengths were measured at the time of the first operation and at the time of autopsy. They were also weighed at weekly intervals during the course of the experiment.

At autopsy the contents of the sella were carefully exposed, inspected with a dissecting microscope, and dissected out and fixed in Champy's fixative. The brain was fixed in formalin. The entire sphenoid bone with a large piece of the attached nasopharynx and neighboring soft tissue of the neck was put in picroformol, and later decalcified. All tissue was serially sectioned and appropriately stained, the contents

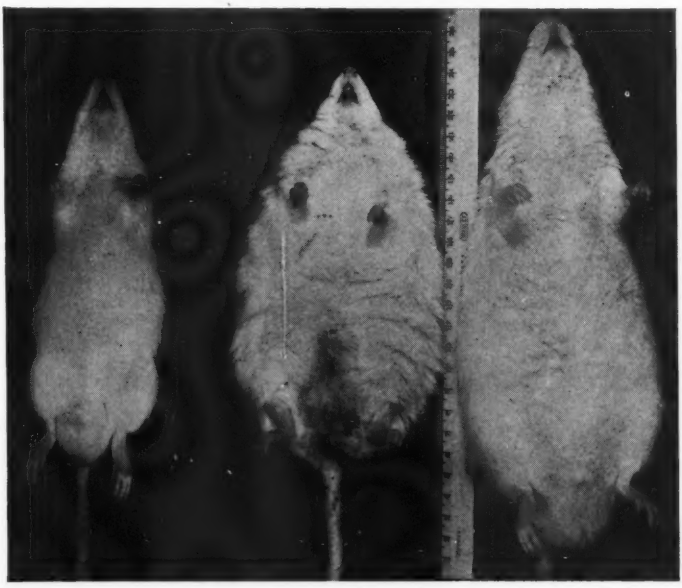

Fig. 1. *Rat Rd2 (center)* IS A COMPLETELY HYPOPHYSECTOMIZED, markedly obese, male dwarf rat having large hypothalamic lesions. On the right is its normal male littermate. On the left is a young normal male (not a littermate) of the same body-length as *Rd2* at the time of operation.

of the sella by the Severinghaus method, the hypothalamus and midbrain with cresyl violet, and the sphenoid block with hematoxylin and eosin. This material was studiously examined for hypophysial remnants.

RESULTS

Six of the 9 animals on which hypophysectomies were carried out were found after a thorough search for pituitary rests to be completely without hypophysial tissue of any sort, save only for the pars tuberalis cells which adhere to the median eminence. The 3 remaining rats retained varying amounts of anterior lobe.

Figure 1 is a picture of *rat Rd2* (male), a markedly obese dwarf rat which proved to be completely hypophysectomized. Also shown, on the right, is *Rd3*, the normal littermate brother of *Rd2*. On the left is a picture of a young normal male, not of the same litter, but of about the same weight and body length as *Rd2* when the latter was operated. The young male was photographed later, the two negatives being then

printed side by side. It serves, in the absence of a more appropriate hypophysectomized littermate, to make clear the tremendous girth of *Rd2*.

As will be seen in the table which summarizes the results, after the usual interval of 3 to 6 weeks, all 9 of the doubly operated animals became fat. An effort is made in the table to express by means of the mathematical relationship existing between the weights and nose-anus lengths of the animals the degree of their adiposity. This formula, the ratio of the cube root of the weight in grams to the body length in centi-

TABLE 1. SUMMARY OF DATA ON HYPOPHYSECTOMIZED ANIMALS HAVING HYPOTHALAMIC LESIONS

Rat Number	Operated or Control	Time of First Operation		Interval Between Operations, days	Time of Autopsy			Obesity	Extent of Hypophysectomy
		Weight, gm.	Nose-anus length, cm.		Weight, gm.	Nose-anus length, cm.	$W^{1/3}/L$		
Rd1♂	O	135	18.0	7	469	24.5	0.317	Medium	Incomplete
Rd2♂	O	108	16.9	7	267	17.5	0.368	Marked	Complete
Rd3♂	C	114	17.3		410	25.6	0.290	Control	
Rd4♀	O	76	15.0	6	176	15.4	0.364	Marked	Complete
Rd5♀	O	99	16.4	6	215	16.9	0.354	Marked	Complete
Rd6♀	C	85	16.0		200	20.5	0.285	Control	
Rd7♀	O*	109	17.3	*	170	19.1	0.290	Absent	*
Rd8♀	O	122	17.3	10	296	18.0	0.370	Marked	Complete
Rd9♀	C	115	17.9		211	21.3	0.279	Control	
Rd10♂	O	123	18.2	10	279	19.2	0.341	Marked	Incomplete
Rd11♂	C	123	18.1		245	22.7	0.276	Control	
Rd12♂	O	116	18.6	10	295	19.2	0.347	Marked	Complete
Rd13♂	C	113	18.2		340	24.6	0.284	Control	
Rd14♀	O	110	16.7	8	229	17.3	0.354	Marked	Complete
Rd15♀	O	95	15.8	8	227	17.9	0.341	Marked	Incomplete
Rd16♀	C	90	16.0		194	20.0	0.289	Control	

* *Rat Rd7* had very asymmetrical hypothalamic lesions, one of which damaged the hypophysis. Because of its poor condition after the first operation, it was not hypophysectomized.

meters, was used by Lee (13) and Cowgill and Drabkin (14) to indicate the 'nutritive state' observed in an individual animal. In the rat colony used by this laboratory the ratios for females range up to a high of about 0.295 ,and for males up to about 0.302. The nutritive index derived from the application of the formula to the obese dwarfs in the table ranges from a low of 0.317 to a high of 0.370, and the majority lie above 0.340.

This range is somewhat higher than that shown by Hetherington and Ranson (2) for a long series of animals with small lesions recently studied. The difference should be laid not to the hypophysectomy, but rather to the fact that the lesions in the hypophysectomized rats were considerably larger. Calculations based on the weight and length figures of the first series of animals reported (1) give indices corresponding to the present ones. That first series, too, was made up of rats with very large hypothalamic lesions. The larger lesions appear to bring about higher levels of obesity.

The skin and hair of these obese dwarfs, particularly those which were completely without hypophysial remnants, closely resembled the skin and hair of ordinary hypophysectomized immature rats. The hair remained rather soft and white, but was perhaps a little sparser. The thyroids, adrenals, and gonads of the completely hypo-

physectomized rats were found at autopsy to be exceedingly small, but they have not been investigated histologically.

COMMENT

The primary conclusion which seems to be justified from these results is that, whatever background rôle the hypophysis may play in the syndrome, the direct mechanism whereby hypothalamic lesions cause adiposity does not operate through the hypophysis. Even the presence of hypophysial tissue, and hence of the hypophysial secretions, is unnecessary in the animal body for hypothalamic lesions to cause obesity. This deduction is supported by other evidence than that covered by the present experiments.

In the first place, simple hypophysectomy, carefully done, does not produce adiposity (4, 5, 15, 16, 17). Part of the significance of this fact for hypothalamic obesity is that it eliminates from consideration the not implausible theory that the hypothalamus may normally control fat metabolism by regulating, via neural or humoral pathways, the output of a 'fat metabolism hormone' by the pituitary. If, on the other hand, the hypophysis were considered to occupy the master rôle, in the sense that its secretions might tone or activate hypothalamic centers which in turn regulate fat metabolism, the same objection would apply: If either theory were true, destroying the hypothalamico-hypophysial partnership at its hypophysial end should be just as effective as at its hypothalamic end. Either procedure would proscribe activity of the hormone. Therefore, hypothalamic adiposity cannot be explained as a secondary hypophysial deficiency syndrome.

Smith (4) reported, of course, that occasionally an hypophysectomized rat may get a little fatter after some months. This is, however, apparently exceptional, and Smith did not mention a search for hypothalamic damage in these animals. The observation has not been duplicated in this laboratory.

Furthermore, were the placing of lesions in the hypothalamus in some way to bring about a release phenomenon in the hypophysis, the latter responding by secreting either excessive amounts of a normal hormone, or some quantity of an abnormal, 'adiposogenic' hormone, then ablation of the hypophysis would circumvent hypothalamic obesity. The present experiments have eliminated this possibility.

Denervation of the pituitary by the cutting of the nervous pathways to it is likewise without effect in the production of obesity, when no hypothalamic injury is involved. In the case of section of the pituitary stalk in several species (18–22) the syndrome has not appeared. It has even been shown (2) that the entire region around the proximal attachment of the stalk, including the median eminence, the arcuate nuclei, and the caudal portions of the suprachiasmatic nuclei can be electrolytically destroyed without precipitating obesity. This procedure would surely interfere with any direct nervous connections between the hypothalamus and the hypophysis.

Section or removal of the cervical sympathetic trunk, or removal of the superior cervical sympathetic ganglion, without any resultant adiposity, is an experimental commonplace.

A third possible neural link between the hypophysis and the brain-stem via the facial and greater superficial petrosal nerves, suggested on the basis of some work by Chorobski and Penfield (23), has failed on examination by Hair and Mezen (24) to afford evidence of any influence upon the gland. In any case, the ability of hypothalamic lesions to cause adiposity in the complete absence of the hypophysis is a fundamental objection to the importance in the disorder of any of these pathways to the hypophysis.

The syndrome appears, rather, to be the sequel of destruction of pathways which travel from the hypothalamus down the brain-stem, and which do not concern the hypophysis at all. The evidence for this point of view is given in detail elsewhere (2) but may be summarized as follows.

Obesity can be produced by fairly large symmetrical lesions which destroy bilaterally the region of the ventromedial hypothalamic nuclei, or interrupt at successively more caudal levels a large number of the descending fibers from the hypothalamic cell groups. Even lesions at the caudal end of the hypothalamus, lying dorsal and lateral to the mammillary bodies, bring about the condition. The pathways have not yet actually been traced into the midbrain.

SUMMARY

Previous work has demonstrated that certain large, bilateral, symmetrical hypothalamic lesions not involving the hypophysis will cause rats to become markedly obese. Lesions of this description were placed in 10 rats, 9 of which were then also hypophysectomized 6 to 10 days after the first operation. After the usual interval of 3 to 6 weeks (following the hypothalamic operation) all of the hypophysectomized animals became progressively fatter until a high degree of adiposity was attained.

When the rats were killed, the hypothalamus and midbrain, the entire contents of the sella, and the sphenoid bone with a large piece of the neighboring ventral soft tissues were prepared for histological study. Examination of these tissues in serial section revealed that 6 animals were completely hypophysectomized, while the remaining 3 had retained small pieces of pituitary at the base of the brain.

Taken together with the evidence that hypophysectomy, stalk section, or destruction of the infundibular region do not cause adiposity, these results are interpreted as indicating that hypothalamic control of fat metabolism, and the dysfunction of that control which leads to adiposity, do not depend upon the hypophysis. Apparently even the presence of the pituitary is non-essential to the appearance of hypothalamic obesity. The response seems to be caused by the severance of pathways which descend from the hypothalamus through the brain-stem.

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BALANCE STUDIES IN HYPOPHYSECTOMIZED AND NORMAL RATS FED ON EQUICALORIC HIGH CARBOHYDRATE AND HIGH FAT DIETS[1]

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EVIDENCE HAS BEEN ADVANCED by Greeley (1) that the tissues of the hypophysectomized rabbit use glucose much more rapidly than do those in the animal with the pituitary intact. This has been supported indirectly by observations of Russell (2) in rats, and also by unpublished data on eviscerated rats in this laboratory. On the other hand, Soskin (3) reports that hypophysectomized, hepatectomized dogs used less sugar then hepatectomized controls. Long, Katzin and Fry (4) have found that hypophysectomized rats are less able to mobilize endogenous protein than are intact animals, and have accounted for the unusual sensitivity of the former group to insulin on the basis of increased glucose utilization coupled with decreased gluconeogenesis.

Since the increase in peripheral glucose utilization is not associated with any evidence of increased metabolic rate, we must assume that there is an associated reduction in the metabolism of some other foodstuff. The normal utilization of endogenous protein in peripheral tissues in the presence of a normally maintained blood sugar is not great enough to account for the difference found even though this were completely suppressed. The question then arises whether *a*), the tissues have lost a considerable portion of their power to oxidize fat; or *b*), their ability to oxidize carbohydrate has been so greatly increased that the rate of burning of fat is indirectly decreased; or finally *c*), whether the carbohydrate disappears because it is converted into some compound which cannot be utilized.

It has been reported by Biasotti (5) that hypophysectomized dogs can be maintained on a high fat diet, and that the bloodsugar level in such animals is normal. However, since Soskin has reported a decreased utilization of glucose in the peripheral tissues of such dogs, it may be that we are dealing here with a species difference. An investigation of the ability of hypophysectomized male rats to maintain themselves on high carbohydrate and high fat diets has, therefore, been carried out, and the relative utilization of each of the three major types of foodstuffs under these circumstances determined.

EXPERIMENTAL PROCEDURE

Male rats of the Sprague-Dawley strain, weighing from 175 to 250 gm. were divided into four groups as nearly alike as possible. *Groups B* and *D* were hypophysec-

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tomized by the parapharyngeal route. *Groups A* and *C* were operated upon in the same way except that the hypophysis was not removed after it was exposed. Six representative rats from the groups were killed at this time and analyzed as a basis for the estimation of the original composition of the other animals. Immediately after the operations the remaining rats were placed in metabolism cages and the urine and feces quantitatively collected. From this time the animals were fed exclusively by stomach tube. The following diets originally described by Reinecke, Ball, and Samuels (6) were fed.

High Carbohydrate Diet	gm.
Dried Egg Albumin	15
Corn Starch	42
Dextrin (from corn)	21
Cane Sugar	21
Osborne-Mendel Salt Mixture	4
Activated Charcoal	5
Cellu-flour	5
Vit. B Concentrate, Squibb	1
Percomorph Oil, 10 drops	
Enough water to make 125 cc.	

High Fat Diet	gm.
Dried Egg Albumin	15
Melted Butterfat	37.8
Osborne-Mendel Salt Mixture	4
Cellu-flour	10
Vit. B Concentrate, Squibb	1
Percomorph Oil, 10 drops	
Enough water to make 125 cc.	

The quantity given was 2 cc. per 100 sq. cm. of body surface twice a day. This was sufficient to maintain the body weight of the control animals at an approximately constant level. It should be pointed out that the animals were fasted before the operations and that the figures given here as the original weight are based upon the weights after surgery, since the control group upon which the original body composition is based had been through this same procedure. *Groups A* and *B* received the high carbohydrate diet while *groups C* and *D* received the high fat diet.

The urine was analyzed for nitrogen, carbohydrate and acetone bodies; the feces, for nitrogen, carbohydrate and fat. Animals were anesthetized 12 and 17 days after surgery and the gastrocnemii were taken for analysis of glycogen and fat. The liver was next removed, divided into two portions, and the large portion analyzed for glycogen and fat while the smaller was analyzed for nitrogen and water. The renal and gonadal fat depots were then dissected. In some cases these were analyzed separately, in others they were combined and analyzed together for fat. The stomach and intestinal tract were each removed and analyzed for carbohydrates, fat and nitrogen. Finally the remainder of the carcass was immediately plunged into boiling water. To this, sufficient sulphuric acid was added to bring the concentration to 10 per cent and the whole boiled for 3 hours. A small aliquot was taken for the determination of total carbohydrate. The remainder was then boiled down and sufficient sulphuric acid added to bring the concentration to 25 per cent and the total volume to 800 cc. The mixture was now boiled under reflux for 12 hours to achieve as complete hydrolysis as possible and the resulting solution with some insoluble humin was transferred to a continuous liquid extractor. The whole was extracted for 16 hours with petroleum ether, the receiving flask containing an excess of alcoholic potassium hydroxide solution. The extract was then analyzed for fat as described later, and the residual solution analyzed for nitrogen. Carbohydrate was estimated by determining the reducing power of the various solutions by the Shaffer-Hartman method, after zinc hydroxide precipitation. In the case of the carcass hydrolyzates, these had to to be first treated with phosphotungstic acid to remove non-sugar reducing substances not precipitated by zinc hydroxide.

Nitrogen was determined by the Kjeldahl procedure. Fat was determined by a modification of the alkali hydrolysis method which will be published in detail elsewhere. Briefly, the sample was completely hydrolyzed with KOH in 50 per cent alcohol. The alcoholic solution was diluted to a given volume and aliquots taken for analysis. The analysis was carried out by strongly acidifying the aliquot and extracting with petroleum ether. The clear petroleum ether extracts were received in a weighed beaker containing a known amount of phenolphthalein. The combined extract in the beaker was diluted with an equal volume of methanol and titrated with potassium methylate in anhydrous methanol. After titration the whole was dried down and the potassium soaps weighed. In the case of the liver the non-saponifiable portion was separated by a somewhat complicated extraction procedure before the titration was made. In the other tissues the non-saponifiable matter was included with the soaps.

Moisture was determined by drying the tissue at 100 to 105° C. until a minimum weight was obtained.

In the hypophysectomized rats the sella turcica was examined under a good dissecting mircoscope to determine the presence of any glandular tissue. The weights of the testes were also checked and the adrenals observed for evidence of atrophy.

RESULTS

Since there was no significant difference between the animals killed after 12 days and those killed after 17 days, all animals of a given type were grouped together.

TABLE 1. ABSORPTION OF FOODSTUFFS DURING BALANCE EXPERIMENTS

Group	Number of Rats	Percentage Absorbed Over Balance Period		
		Fats	Carbohydrate	Nitrogen
A. Carbohydrate-fed, controls	9		98.6	81.9
B. Carbohydrate-fed, hypophysectomized	6		98.3	76.4
C. Fat-fed, controls	12	93.2		86.6
D. Fat-fed, hypophysectomized	7	89.1		86.5

Table 1 gives the average amounts of fat, carbohydrate, and protein absorbed by the animals in the various groups for the entire experimental period. There was no statistically significant difference in the utilization of any of these, although the evidence suggests that there may have been a slight decrease in the absorption of protein in the carbohydrate-fed hypophysectomized rats.

While this shows that the various foodstuffs are digested and absorbed in the hypophysectomized animal rapidly enough to be normally utilized, the data in table 2, which are taken from an experiment in which a given amount of food was fed two hours before the death and dissection of the animals, indicate that passage from the stomach and absorption from the gut are apparently slower in the hypophysectomized rats.

Since the hypophysectomized rats do not apparently waste any greater amount of foodstuffs than similar intact animals when all groups are fed sufficient amounts of food to maintain the body weights of the controls, the next question of interest is whether the different foodstuffs are oxidized in a normal manner. In table 3 the sources of energy of the different groups are given as calculated from the difference between intake and excretion of the different foodstuffs plus the changes in body composition.

TABLE 2. DISTRIBUTION OF FOODSTUFFS TWO HOURS AFTER MEAL BY STOMACH TUBE

Groups	A Carbohydrate-fed, Controls	B Carbohydrate-fed, Hypophysectomized	C Fat-fed, Controls	D Fat-fed, Hypophysectomized
Number of Rats	8	5	9	5
Fats, percentage:				
in stomach			22.4	28.4
in small intestine			7.2	10.5
absorbed			70.4	61.1
Carbohydrate, percentage:				
in stomach	25.0	33.6		
in small intestine	4.8	5.8		
absorbed	70.2	60.6		
Nitrogen, percentage:[1]				
in stomach	37.5	53.0	32.2	46.6
in small intestine	21.3	26.8	22.4	24.9
absorbed	41.2	20.2	45.4	28.5

[1] This nitrogen includes the nitrogen of the digestive juices.

In the quantities fed, protein was apparently utilized with equal readiness by all groups. Fat, when fed in the diet, was oxidized by the hypophysectomized rats as readily as by the controls, and there was no apparent reduction in the rate of total metabolism when based on the fat balance. When carbohydrate was fed to the hypophysectomized rats, however, storage of fat was increased approximately 20 per cent over the control group. The total energy utilization of these animals varied significantly from those of the other three, being 18 per cent lower than their mean.

If fat were being stored, one would expect an increase in body weight. Both groups of hypophysectomized rats lost a small amount of weight, however, while the control groups gained 2.5 to 2.9 per cent during the feeding period. The reasons for this are shown in table 4 in which the original and final composition of the rats is compared on the basis of 100 gm. of original weight. The values in the column headed 'H_2O, Salts' were obtained by subtracting the amounts of carbohydrate, fat, and protein from the equivalent proportion of the total weight. Under 'original composition' the sum of the four columns for each group will equal 100 per cent; under the 'final composition' the sum of the four columns will be greater or less according to the weight gained or lost.

There are two significant changes in composition. The decreased energy utilization of the carbohydrate-fed hypophysectomized rats is accounted for by a 3.77 per cent

TABLE 3. ENERGY CONSUMPTION OF RATS ON DIETS CONTAINING 15 PER CENT PROTEIN
(*Calories per 100 sq. cm. of original body surface per day*)

Number and Type of Animal	Carbohydrate	Fat	Protein	Total
6 Hypophysectomized rats, high carbohydrate diet	10.29 ±.06[1]	−1.92±.66	1.22±.05	9.58±.70
9 Control rats, high carbohydrate diet	10.35 ±.09	0.35±.37	1.25±.40	9.58±.70
6 Hypophysectomized rats, high fat diet	−0.022±.006	10.12±.33	1.44±.08	11.95±.40
9 Control rats, high fat diet	−0.045±.004	10.56±.46	1.31±.07	11.56±.31

[1] Standard-Error.

TABLE 4. CHANGE IN BODY COMPOSITION ON DIET CONTAINING 15 PER CENT OF CALORIES AS PROTEIN
(*Gram/100 gm. of original weight*)

Number and Type of Animal	Original Composition[1]				Final Composition				Change in Composition				
	Carbohydrate	Fat	Protein	H_2O and salts	Carbohydrate	Fat	Protein	H_2O and salts	Weight	Carbohydrate	Fat	Protein	H_2O and salts
6 Hypophysectomized rats, high carbohydrate diet	0.37	9.55	17.77	72.3	0.42	13.32	18.53	66.5	−1.2	+0.05	+3.77	+0.76	−5.8
9 Control rats, high carbohydrate diet	0.36	8.89	18.01	72.7	0.45	8.43	19.03	75.1	+2.9	+0.09	− .46	+1.02	+2.4
6 Hypophysectomized rats, high fat diet	0.37	9.55	17.77	72.3	0.26	11.08	18.93	66.1	−3.6	−0.11	+1.53	+1.16	−6.2
9 Control rats, high fat diet	0.36	8.89	18.01	72.7	0.36	10.11	19.76	72.3	+2.5	0	+1.22	+1.75	−0.4

[1] Based on analysis of rats from same groups killed at beginning of experiment.

increase in the amount of body fat. But this gain in weight was more than offset by a 5.8 per cent loss in 'H_2O, Salts.' A similar loss, 6.2 per cent, in this same group of constituents was shown by the fat-fed hypophysectomized animals. Since the tibiae of all of the groups at the end of the experiments had similar average weights, this decrease was probably due to loss of water. This, therefore, accounts for the fact that the hypophysectomized animals lost weight on a food intake which maintained the weights of the control rats, even though they were utilizing the food equally well and their metabolic rates were not higher. This also seems to be the only difference from the controls which is shown by both hypophysectomized groups. We can therefore conclude that this is the only factor measured which depends upon the pituitary gland irrespective of the diet.

The greater fat formation in the hypophysectomized carbohydrate-fed rats is not associated with a greater liver fat content. In table 5 the amounts of fatty acids per 100 gm. of original weight in the liver and peripheral tissues are compared. The two hypophysectomized groups show definitely lower liver fat content than the controls. The difference is particularly striking in the carbohydrate-fed groups, in which the hypophysectomized animals have the lowest liver fat and the highest body fat of all. The lipid material in the livers of these rats could probably be accounted for almost entirely by the phospholipid and non-saponifiable fractions. The controls have the lowest peripheral fat content and an intermediate content of fat in the liver, slightly higher than the fat-fed hypophysectomized group.

The livers of the hypophysectomized rats are smaller, and the decrease in active liver tissue may interfere with the storage and mobilization of fat. However, the

TABLE 5. DISTRIBUTION OF FAT BETWEEN LIVER AND BALANCE OF CARCASS IN HYPOPHYSECTOMIZED AND CONTROL RATS ON EQUICALORIC RATIONS

Group	Number of Rats	Liver Weight, Gm./100 Gm.	Liver Fat, Gm./100 Gm.	Carcass Fat, Original Body Weight
A. Fat-fed, controls	9	3.61	.585	9.53
B. Fat-fed, hypophysectomized	6	3.18	.245	10.80
C. Carbohydrate-fed, controls	9	3.85	.291	8.14
D. Carbohydrate-fed, hypophysectomized	6	3.44	.098	13.23

difference is not entirely accounted for on this basis since the percentages of fat in the livers of the deficient groups are lower than in their corresponding controls.

DISCUSSION

It would seem from these results and those of others, that one must distinguish sharply between endogenous and exogenous sources of at least two of the foodstuffs: fat and protein. The work of others has shown that endogenous protein is not so readily mobilized in the absence of the pituitary gland. All of this work has been done in animals on diets *ad libitum*, whereby the hypophysectomized rat considerably reduces its intake. The matter of lower food intake has been controlled in some studies by the system of paired feeding, and the differences in nitrogen output on fasting have still been apparent. However, it must be remembered that this does not assure a similar proportion of carbohydrate, fat and protein in the body tissues even though the total weights might be equal. The results of this study would indicate that on such a regime there might well be an increased amount of peripheral fat in the hypophysectomized animals and a lower protein. The decrease in liver protein is particularly significant since the work of Addis (7) indicates that this is the source of the animal's most labile supply. However, since there seems to be a differential effect on the mobilization of body fat, the same may be true for the muscle protein. Under these circumstances, with less of the labile liver protein to convert into carbohydrate, it is not surprising that hypophysectomized rats should develop hypoglycemia more quickly than normal rats under identical feeding conditions, particularly when there has been a long period of low food intake.

It would seem that the fact that the carbohydrate-fed hypophysectomized animals were the only ones to show a lowered metabolic rate indicates again that carbohydrate utilization is most seriously disturbed in animals without the pituitary. It is obvious from the work of Addis, however, that the rapid disappearance of glucose in such animals is not due to an inability to use the other foodstuffs. These results would indicate that it is not even preferentially used, since, where fat was abundantly supplied, the carbohydrate stores of the body were as high as in the controls. The most probable explanation is that carbohydrate is accumulated as fat in the tissues of such animals with unusual rapidity. This may not be because the conversion is more rapid but, since at all times there appears to be a continuous utilization of fat according to Schoenheimer (8), the mobilization of fat or the use of some intermediate is interfered with and the net result is an apparent more rapid accumulation of the body fat. Two observations support an interference with fat metabolism. Best (9) has shown that in animals treated with anterior pituitary extracts, fat is mobilized more rapidly than in untreated controls. Second, our own data demonstrate that in the presence of larger fat stores there is less fat in the livers of hypophysectomized rats, an organ which normally increases in fat content when fat is being utilized in large quantity.

The rapid formation, or slow mobilization, of fat may be associated with tissue changes which result in the early reduction in total metabolism. In the fat-fed animals sufficient fat is available in the liver to maintain the metabolic rate for this period.

Changes in water metabolism due to the pituitary gland are apparently unconnected with differences in energy metabolism. Other work has indicated that this is probably affected primarily by the posterior lobe.

SUMMARY

1. Both hypophysectomized and control adult male rats were fed by stomach tube on sufficient food intake to maintain body weight. One group of each was given

a diet of which 85 per cent of the calories were provided by carbohydrate, while the others were given a diet containing 85 per cent of the calories as fat. All animals were able to absorb the diets efficiently, although the hypophysectomized rats apparently did not absorb them so rapidly.

2. The metabolic rates of all the groups, based on the balances, was the same within experimental error except that of the carbohydrate-fed hypophysectomized animals which was 18 per cent below the others. This lower rate was associated with an increased amount of peripheral fat.

3. The only other significant change in total body composition during the experiment was a loss of water from the hypophysectomized rats. This was true for both fat-fed and carbohydrate-fed groups.

4. There was less fat in the livers and more in the peripheral tissues of the hypophysectomized rats than in the controls. This was particularly marked in the carbohydrate-fed animals. It would seem that fat once stored in the peripheral tissues is not readily utilized.

5. Apparently within 17 days after hypophysectomy the ability to burn fat has not been decreased nor has the tendency to oxidize carbohydrate been significantly increased. Carbohydrate seems to be readily converted into fat.

6. An hypothesis is advanced which would explain many of the metabolic phenomena on the basis of a change in the rate of conversion of carbohydrate to fat and in the utilization of stored fat.

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EFFECT OF DIET ON GLUCOSE TOLERANCE AND LIVER AND MUSCLE GLYCOGEN OF HYPOPHYSECTOMIZED AND NORMAL RATS[1,2]

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THE HYPOPHYSECTOMIZED RAT on an *ad libitum* or force-fed diet has been shown to exhibit an abnormal glucose tolerance curve (1, 2, 3). During the period of rapid glycogen formation the rate of removal of glucose from the blood is less than normal, but during the later period when glycogenolysis maintains the blood-sugar level in normal animals, the blood-sugar level of the hypophysectomized rat continues to fall. This appears to be the result of the rapid removal of glucose from the blood stream by the peripheral tissues as demonstrated by Greeley in the hypophysectomized rabbit (4).

In balance studies on rats fed high carbohydrate and high fat diets by stomach tube for 10 to 17 days after hypophysectomy, it was found that while the carbohydrate-fed rats showed a low metabolic rate and lower carbohydrate stores than control animals on the same diet, the fat-fed animals still had a normal metabolic rate and a normal amount of carbohydrate in their bodies (5). The carbohydrate-fed hypophysectomized rats had increased amounts of body fat together with low liver fats. It was suggested that a possible reason for the rapid disappearance of glucose in the peripheral tissues of the hypophysectomized rats was its rapid conversion to fat which could not be normally mobilized. This hypothesis had been previously mentioned by Greeley although he thought that his evidence was against this. To determine whether the previous diet exerted any greater influence on the disappearance of glucose from the blood stream in hypophysectomized than in control rats, we fed groups of such animals on high fat and high carbohydrate diets having the same caloric proportion of the same kind of protein. Intravenous glucose tolerance tests were then made and the difference determined.

METHODS

Adult male rats of the Sprague-Dawley strain were hypophysectomized by the parapharyngeal route. Litter mates of these animals were operated in the same way except that the pituitary gland was not aspirated. The animals were then placed on the following diets (6).

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[2] Assistance in the preparation of these materials was furnished by the personnel of the Works Project Administration, Official Project No. 165-1-71-124, Sub-Project #355.

High Carbohydrate Diet	gm.
Dried Egg Albumin	15
Corn Starch	42
Dextrin (from corn)	21
Cane Sugar	21
Osborne-Mendel Salt Mixture	4
Activated Charcoal	5
Cellu-flour	5
Vit. B Concentrate, Squibb	1
Percomorph Oil 10 drops	
Enough water to make 125 cc.	

High Fat Diet	gm.
Dried Egg Albumin	15
Melted Butterfat	37.8
Osborne-mendel Salt Mixture	4
Cellu-flour	10
Vit. B Concentrate, Squibb	1
Percomorph Oil 10 drops	
Enough water to make 125 cc.	

The diets were fed by stomach tube in sufficient quantity to maintain the body weight of the control animals, 2.0 cc. per 100 sq. cm. of body surface twice a day. After 2 weeks on the diet, the animals were fasted for 24 to 30 hours and glucose tolerance tests made as described by Samuels et al. (3). A 50 per cent glucose solution was injected into the saphenous vein, 125 mg. of glucose being administered per 100 gm. of body weight. Samples of blood were then withdrawn from the tail vein at intervals of 10, 20, 30, 60, 120, 180 and 240 minutes after the glucose injection. One hour after the last sample was taken the animals were anesthetized with amytal and the liver and gastrocnemius muscle were removed and immediately dropped into hot 30 per cent potassium hydroxide for determination of glycogen and fat.

Glucose was determined by the Jeghers and Myers modification of the Folin ferricyanide method (7). Glycogen was estimated according to Good, Kramer and Somogyi (8). Fat was determined on the supernatant fluid and washings from the glycogen by acidification, extraction, titration and weighing the soaps.

RESULTS

In figure 1 are shown the average sugar tolerance curves for the four groups of

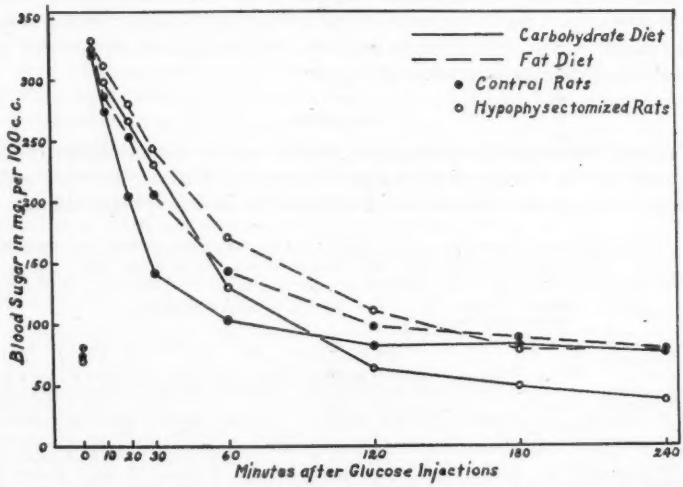

Fig. 1. Average intravenous glucose tolerance curves of control and hypophysectomized rats on high carbohydrate and high fat diets. At zero time 0.125 gm. of glucose per 100 gm. of body weight was injected into the saphenous vein.

TABLE 1. LIVER AND MUSCLE GLYCOGENS OF FASTED HYPOPHYSECTOMIZED AND CONTROL RATS PREVIOUSLY MAINTAINED ON EQUICALORIC AMOUNTS OF HIGH CARBOHYDRATE OR HIGH FAT DIETS

	Number Rats	Liver Glycogen, mg./100 gm. of body weight	Muscle Glycogen, mg./100 gm.
A. Carbohydrate-fed controls	11	2.5 0.3–10.3	478 410–462
B. Fat-fed controls	16	19.7 2.3–36.5	444 312–631
C. Carbohydrate-fed, hypophysectomized	8	<0.1 <0.05–0.16	176 66–258
D. Fat-fed, hypophysectomized	10	9.1 0.1–29.7	443 283–630

animals. Table 1 gives the mean values of the blood sugars for the different time periods together with the standard errors and the evidence of significance in the differences between the various groups. The control animals on the carbohydrate diet show a significantly more rapid drop during the period of sugar removal than do the fat-fed controls. However, they level off at the same point during the period of glycogenolysis. The hypophysectomized animals show no significant difference due to diet during the period of glycogenesis. However, the carbohydrate-fed group was not able to maintain blood-sugar levels during the period of glycogenolysis. Some rats went into hypoglycemic convulsions. None of the fat-fed hypophysectomized rats had convulsions and in most cases they tended to maintain their blood-sugar levels better. As shown in table 2, the glycogen content of the livers was less than in the fat-fed controls but greater than in the carbohydrate-fed controls. Muscle glycogens were approximately normal in the fat-fed hypophysectomized group while in the similar carbohydrate-fed animals the values were abnormally low, as found by other workers (9). There were some of the fat-fed rats, however, which were not able to maintain their blood sugars any better than the carbohydrate-fed group. These also had low muscle and liver glycogens.

DISCUSSION

The best explanation of these results would seem to be that neither the carbohydrate-fed nor fat-fed hypophysectomized rats were able to mobilize their endogenous stores of energy at a normal rate. Exogenous fat, however, could take the place

TABLE 2. INTRAVENOUS GLUCOSE TOLERANCE STUDIES IN FASTED HYPOPHYSECTOMIZED AND CONTROL RATS, PREVIOUSLY FED EQUICALORIC HIGH CARBOHYDRATE AND HIGH FAT DIETS

Group	Number of Rats	Before Injection	After Glucose Injection						
			10′	20′	30′	60′	120′	180′	240′
A. Carbohydrate-fed, controls	12	74±2.9	274±7.8	203±7.6	140±3.0	103±3.0	82±2.0	83±3.3	74
B. Fat-fed controls	16	82±2.3	287±8.4	253±5.5	205±9.5	143±6.8	98±3.0	88±3.0	80
C. Carbohydrate-fed, hypophysectomized	8	70±8.7	298±9.6	266±10.5	231±12.8	130±17.4	63±4.2	49±5.5	38
D. Fat-fed, hypophysectomized	12	82±3.4	312±8.7	280±11.2	244±9.9	171±15.0	111±9.4	79±5.0	77
$\frac{\text{Difference}}{\text{S.E. Difference}}$ A and B			1.1	5.3	6.3	5.4	4.4	1.1	
$\frac{\text{Difference}}{\text{S.E. Difference}}$ C and D					.8	1.8	4.7	4.0	

of carbohydrates as fuel. Because of slower absorption and greater amounts of available fat in the liver, the fat-fed hypophysectomized rats were able to conserve their glycogen stores for a longer time and thus could maintain their blood-sugar levels during periods of fasting. The carbohydrate-fed rats did not store so much fat in the liver but, instead, laid it down as peripheral body fat. The glycogen and fat of the liver were available for use, but there was interference with the normal replenishment of these from the body stores of fat and protein.

The effect was on fat as well as protein since there were ample stores of the former available in the carbohydrate-fed hypophysectomized rats (5) at a time when the liver content was low and the glucose was being drained from the blood and the tissues. Since there is ample evidence that the metabolism of fat and protein are intimately connected, it may well be that the effect of the pituitary is on reactions in which both are involved.

The influence of the high fat diet on the rate of disappearance of liver glycogen during subsequent fasting was not limited to the hypophysectomized animals. The data in this experiment bear out findings previously reported (10), that glycogen disappears more slowly during fasting after a high fat-low protein diet than after a high carbohydrate-low protein diet. It would seem, then, that the adjustment of the animal to a high rate of fat metabolism and the presence in the liver of considerable amounts of fat make it possible for the rat to continue to burn large amounts of fat during fasting and thus to conserve glycogen stores. In the normal rat this process is continued by replenishment of the liver fat from peripheral fat stores. In the hypophysectomized rat this process is decreased and the animal eventually develops hypoglycemia.

SUMMARY

Hypophysectomized rats fed adequate amounts of diets in which 85 to 90 per cent of the calories were supplied by either carbohydrate or fat were given glucose tolerance tests after a 24-hour fasting period. Both showed the delayed removal during the glycogenic period, but the fat-fed rats were better able to maintain their blood-sugar levels during the period of glycogenolysis. Some were not able to maintain the blood-sugar level as well as the normal animals, however. The greater ability to maintain the blood-sugar levels was associated with better liver and muscle glycogen supplies at the end of the experiment.

It is considered that this is additional evidence that the metabolic disturbance in hypophysectomized rats is not on their ability to use any one of the foodstuffs as long as it is available in proper form in the liver and circulation. The fundamental disturbance is in the mobilization of endogenous stores, both of protein and fat.

Adjustment of either a normal or hypophysectomized rat to a high fat diet before fasting increased the relative rate of utilization of fat and decreased carbohydrate utilization during a subsequent fasting period.

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NORMAL ENDOCRINE GLAND WEIGHTS OF FEMALE RATS OF THE SPRAGUE-DAWLEY STRAIN THROUGHOUT THE GROWTH PERIOD AND ADULT LIFE[1]

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SINCE THE SPRAGUE-DAWLEY strain of albino rat has come into widespread use as a laboratory animal, it has seemed desirable to have available normal standards for this strain comparable to those prepared by Donaldson (1) for the Wistar strain and by Freudenberger (2) for the Long-Evans and Wistar strains.

In the course of an investigation previously reported (3), we had the opportunity of obtaining data on a large number of normal females of the Sprague-Dawley strain, ranging in age from 2 to 40 weeks.[4] Included were body weight, body length and weights of the pituitary (all lobes), thyroid, thymus, adrenals, ovaries and uterus. These data have been organized and are here presented with the thought that they may serve as useful standards of reference for endocrine gland weights of the female of the Sprague-Dawley strain of albino rat.

Source of the strain. Sprague-Dawley, Inc. has furnished the following brief history of their strain: "Our strain started originally with a white hybrid hooded male mated to his female offspring for seven successive generations. After his death, his white offspring were inbred in a number of different lines from which the best ten were combined. Selection was made to retain or acquire the characteristics of high lactation, rapid growth, vigor, temperament, and high resistance to arsenic trioxide. Attempts were made to increase susceptibility to various cancers but these resulted in loss of vigor and were abandoned."

Diet. All animals used in this investigation were obtained directly from the Sprague-Dawley colony where they had been maintained in a healthy condition on the following diet:

Corn	23	Alfalfa	7
Wheat	28	$CaCO_3$	0.5
Oil meal	13	NaCl	1
Meat scraps	10	Lard	2
Milk powder	16		

Before autopsy they were kept in our laboratory for varying periods. The diet there consisted of Purina Dog Chow, supplemented by greens several times weekly and codliver oil once a week.

Methods. Groups of 20 to 57 females were killed at ages ranging from 14 days to 40 weeks. Immediately before autopsy, they were anesthetized with ether to permit

[1] This investigation was supported in part by a special grant from the Wisconsin Alumni Research Foundation.

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[4] Most of the animals used in this study were gratuitously furnished by Sprague-Dawley, Inc., Madison, Wisconsin.

accurate body length measurement (tip of nose to base of tail). After partial recovery, they were killed by rapid decapitation and bled completely. All glands were cleanly dissected and rapidly weighed on a Roller-Smith torsion balance. All rats beyond puberty were in proestrus at time of autopsy, as determined by vaginal smear. Intrauterine fluid was expressed before weighing the uterus.

Two hundred and thirty-four rats were autopsied in this study. The mean and standard error of the mean were computed for each age group. The latter was determined from the formula:

$$\sigma \text{ mean} = \frac{\text{standard deviation}}{\sqrt{n}} \qquad \frac{\sqrt{\frac{\sum x^2}{n-1}}}{\sqrt{n}}$$

Results. Table 1 summarizes the results for each age group. In figures 1 to 15 all individual weights are plotted against body weight and body length. No attempt is

TABLE 1. MEANS AND STANDARD ERRORS OF THE MEAN FOR BODY WEIGHT AND LENGTH, AND WEIGHTS OF VARIOUS ENDOCRINE GLANDS FROM NORMAL FEMALE RATS OF THE SPRAGUE-DAWLEY STRAIN.

Age at autopsy	14 days	21 days	25 days	5 weeks	7 weeks	12 weeks	16 weeks	24 weeks	40 weeks
Number of rats	21	21	25	20	57	25	25	20	20
Body weight, grams	23.1± 0.4	35.2± 0.5	46.8± 0.5	75.6± 1.2	112± 1.3	161± 1.6	192± 2.8	216± 2.9	227± 4.8
Body length, centimeters	9.0± 0.04	10.7± 0.06	11.9± 0.05	14.4± 0.07	16.3± 0.07	18.7± 0.08	19.7± 0.10	20.6± 0.12	21.3± 0.12
Kidney weight, gram	0.255± 0.006	0.391± 0.006	0.537± 0.007	0.724± 0.016	1.058± 0.014	1.231± 0.021	1.351± 0.026	1.419± 0.036	1.573± 0.031
Pituitary weight, mg.	1.7± 0.04	2.0± 0.06	2.6± 0.07	3.3± 0.07	5.8± 0.39	7.4± 0.21	10.7± 0.35	13.2± 0.49	16.1± 0.60
Thyroid weight, mg.	3.1± 0.10	4.3± 0.13	No data	4.8± 0.16	7.0± 0.18	8.2± 0.22	9.2± 0.29	10.6± 0.51	12.0± 0.20
Thymus weight, mg.	97± 4.1	151± 7.0	151± 4.7	217± 17.8	305± 7.0	294± 12.3	247± 11.1	173± 8.8	110± 6.3
Adrenal weight, mg.	6.6± 0.20	13.1± 0.43	14.2± 0.33	18.4± 0.50	27.6± 0.58	41.8± 0.77	52.0± 1.15	51.1± 1.62	53.5± 1.48
Ovary weight, mg.	3.1± 0.22	10.9± 0.33	13.2± 0.36	16.1± 0.60	24.7± 1.21	36.3± 1.65	46.8± 1.44	48.4± 1.73	53.3± 2.33
Uterus weight, mg.	14.7± 0.6	20.0± 0.6	19.7± 0.4	No data	120± 8.7	No data	436± 12.4	549± 21.2	673± 35.3

made to fit these data to theoretical curves. The rate of growth of the several organs and the extent of variation can best be appreciated by direct inspection of these all-point graphs. The high degree of correlation between body weight and length (fig. 1) permits the use of either as a satisfactory basis for comparison of gland weights.

In 1932, Freudenberger (2) conducted an extensive investigation comparing the Long-Evans and Wistar rats. He has kindly permitted us to reproduce a portion of his data here in order that a further comparison may be made between the females of those strains and the Sprague-Dawley strain. In table 2, Freudenberger's 3-week, 12-week and 1-year-old females are compared to our 3-week, 12-week and 40-week-old Sprague-Dawley females, respectively. The oldest groups are included in spite of the difference in age, because the body length and certain of the gland weights correspond quite closely. If due allowance is made for this age discrepancy and for the fact that conditions such as diet were not identical in the two studies, reasonable comparison may be made. In the interest of simplicity only the means are shown in table 2.

TABLE 2. COMPARISON OF WISTAR (W), LONG-EVANS (*L-E*) AND SPRAGUE-DAWLEY (*S-D*) STRAINS. The data for the first two strains are taken from tables 4, 6 and 8 of Freudenberger (2). Only the means are shown, and all data are from females only.

Age at autopsy	3 weeks			12 weeks			1 year[1]		
Strain	W	L-E	S-D	W	L-E	S-D	W	L-E	S-D
Body weight, gram	38.0	38.6	35.2	157.1	176.4	161.0	254.9	282.6	227.0
Body length, cm.	11.1	11.0	10.7	18.1	18.9	18.7	21.6	21.9	21.3
Kidney weight, gram	0.545	0.631	0.391	1.343	1.527	1.231	2.193	2.224	1.573
Pituitary weight, mg.	2.0	2.1	2.0	8.4	8.2	7.4	14.9	14.0	16.1
Thyroid weight, mg.	8.8	8.1	4.3	25.4	29.4	8.2	33.5	41.0	12.0
Thymus weight, mg.	214.6	116.5	154.0	344.9	276.0	294.0	117.0	81.0	110.0
Adrenal weight, mg.	12.1	13.7	13.1	40.5	53.3	41.8	51.3	57.8	53.5
Ovary weight, mg.	16.5	14.0	11.0	56.0	54.7	36.3	62.7	66.6	53.3
Uterus weight, mg.	24.6	19.0	20.1	359.0	292.0		673.0	447.0	673.0

[1] The Sprague-Dawley group in the last column was only 40 weeks old. See text for explanation.

DISCUSSION

No attempt is made statistically to evaluate the various differences revealed in table 2. It is readily apparent, however, that several of these differences are large enough to be considered as true strain variations. The data show that the Sprague-

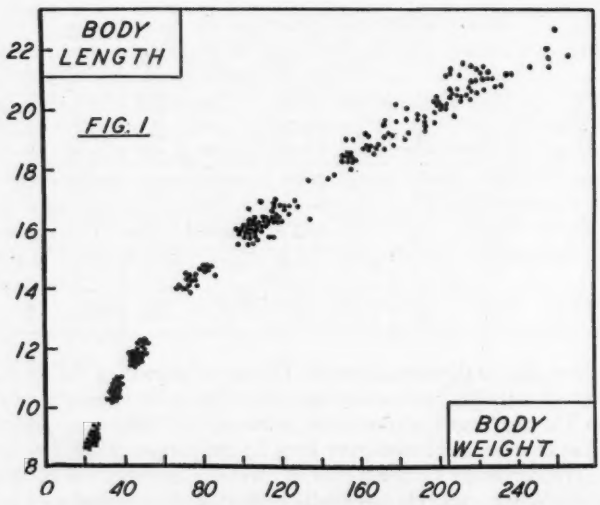

Fig. 1. BODY LENGTH IN CENTIMETERS PLOTTED AGAINST BODY WEIGHT IN GRAMS. In this and all subsequent figures, each point represents one animal. All data are from females.

Dawley females differ from either or both of the other strains especially in the weights of the pituitary (at 12 weeks), thyroid (all ages), thymus (3 weeks), adrenals (12 weeks), ovaries (all ages), and kidneys (all ages). It is noted, furthermore, that although the body length of Sprague-Dawley females at 40 weeks is nearly the same as in the Wistar and Long-Evans females one year old, the body weight is considerably

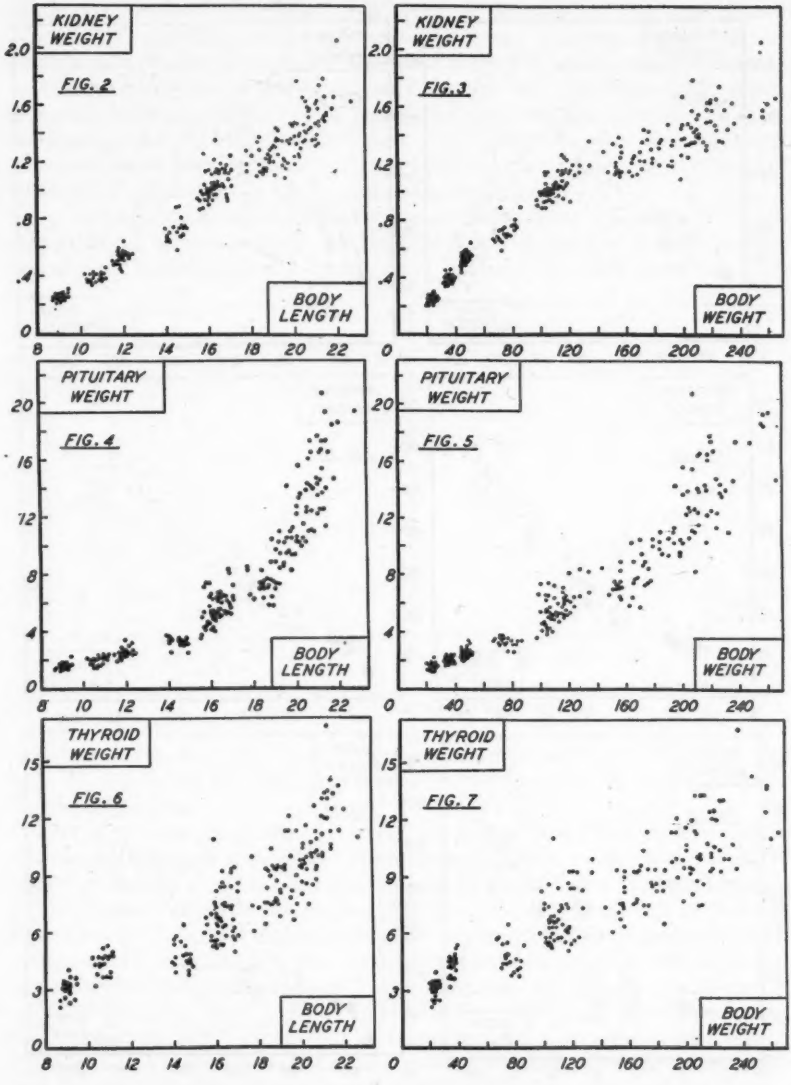

Fig. 2–7. ORGAN WEIGHTS PLOTTED AGAINST BODY LENGTH (*cm.*) AND BODY WEIGHT (*gm.*). Kidney weight is expressed in grams and all other gland weights are expressed in milligrams.

less than in the latter two strains. In the younger groups, this difference does not exist. The consistently smaller size of the kidneys in the Sprague-Dawley strain is worthy of emphasis.

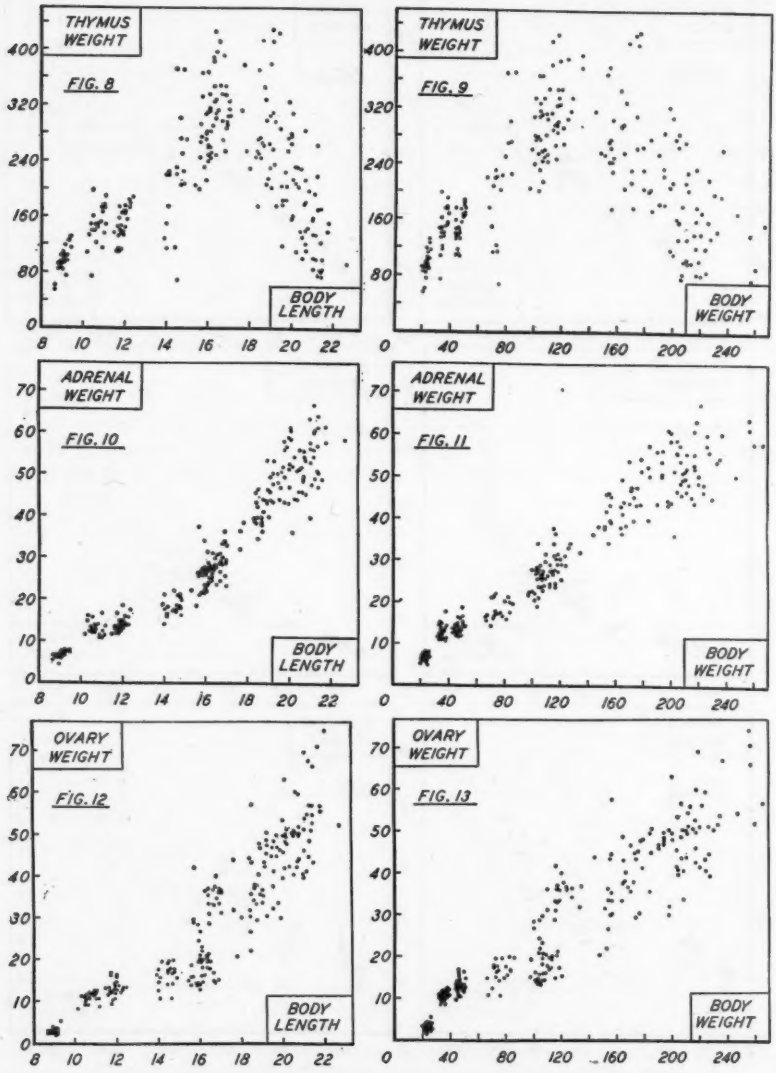

Fig. 8–13. Organ weights plotted against body length (cm.) and body weight (gm.). Gland weights are expressed in milligrams.

Most outstanding is the remarkably lower weight of the thyroid gland in the Sprague-Dawley rats. At 3 weeks it is only one-half as heavy and in the oldest group only one-third as heavy as in the Wistar and Long-Evans strains. So marked is this difference that further verification was sought. Thyroid weight data from over 200

female rats were kindly furnished to us by Dr. R. K. Meyer of the University of Wisconsin Zoology Department (4), whose rat colony was originally of Sprague-Dawley stock, further in-bred for a number of years. His figures correspond closely to those presented here, indicating that small thyroids are a true strain characteristic. It is improbable that iodine supply played any significant rôle in producing these differences, since both Minnesota (where Freudenberger's work was done) and Wisconsin are in the same so-called 'goiter belt,' and since neither we nor Freudenberger made any attempt to supply extra iodine in the diet.

In addition to serving as norms for females of the Sprague-Dawley strain, the data presented here are useful in revealing the degree of variation in organ weights encountered in normal animals reared under ordinary laboratory conditions. The

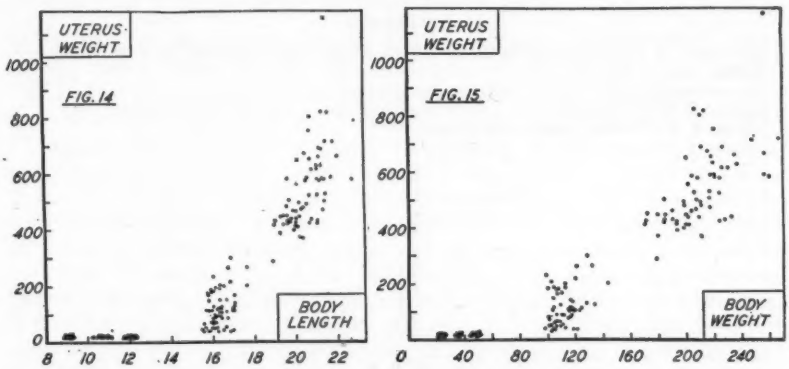

Fig. 14 and 15. ORGAN WEIGHTS PLOTTED AGAINST BODY LENGTH (cm.) AND BODY WEIGHT (gm.). Organ weights are expressed in milligrams.

widest scatter is seen in the thymus weights, where, for example, the range is from 75 to 370 mg. in rats weighing 70 to 90 gm. The kidneys show the least variation of the organs weighed.

In the previous work referred to above (3), we had been especially interested in the changes occurring at puberty. Fifty-seven animals were autopsied at 7 weeks of age at which time about half had reached puberty as manifested by vaginal opening and enlargement of uterus and ovaries. The data from this group may be found in figures 1 to 15 at the level of about 16 cm. for body length and about 110 gm. for body weight. Significant changes in the rate of growth are seen about the time of puberty in nearly all of the glands studied. Greatest alterations occur in the case of the hypophysis (fig. 4, 5), thymus (fig. 8, 9), ovaries (fig. 12, 13), and the uterus (fig. 14, 15). Apology is offered for the lack of uterine weight data in the 5 and 12-week-old groups. These animals were autopsied before the final form of the investigation was defined. Although the uteri were not weighed in these groups, those of the 5-week-old animals were uniformly infantile in appearance, while those of the 12-week-old group were all distended with fluid to about the same degree.

It is probably unnecessary to emphasize that environmental factors such as diet and temperature affect the rate of growth and development. For this reason the data should not be regarded in an absolute sense. However, if gland weights are expressed in terms of body length or body weight, reasonable correspondence would be expected under average laboratory conditions.

SUMMARY

Data are presented on normal body length, body weight, and weights of the pituitary, thyroid, thymus, adrenals, ovaries and uterus of 234 females of the Sprague-Dawley strain of albino rat, ranging in age from 14 days to 40 weeks. All-point graphs are shown in which weights of glands are plotted against body length and weight. Comparison is made between certain age groups of this strain and corresponding age groups of females of the Wistar and Long-Evans strains, the data of Freudenberger (1932) being used. Of the many differences noted, most striking is the smaller size of the thyroid in the Sprague-Dawley strain. These data are submitted with the thought that they may serve as standards of reference for female endocrine gland weights of the widely-used Sprague-Dawley strain.

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GROWTH IN SILVER DWARF MICE, WITH AND WITHOUT INJECTIONS OF ANTERIOR PITUITARY EXTRACTS

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WE HAVE PREVIOUSLY SHOWN (1) that, when dwarf mice from the purebred Silver strain are used throughout, a recombination of isoelectrically insoluble and soluble fractions from extracts of the anterior pituitary yields potentiation beyond their additive growth-stimulating effects by as much as 10 to 20-fold. The significance of this conclusion has been questioned by Boettiger and Osborn (2) who present data for untreated dwarf mice that show as much growth as most of our treated dwarfs. Since the statement had been repeatedly published that, under the conditions of our laboratory, dwarf mice from the pure-bred Silver strain do not show any significant growth after weaning, it was not deemed necessary to publish the records for the untreated dwarfs that were actually carried in every group of treated dwarfs as controls on the daily variations in food and other environmental influences. Besides meeting the above criticism, by presenting the unpublished records for controls, we now offer a considerable mass of data for body weights of untreated Silver dwarfs. In addition, we present supplementary experimental data which show the fit of recombined anterior pituitary fractions to the same regression line previously used for relating dosage and response of the separate fractions.

Before considering the data attention will be called to a misconception that appears to lie behind the above criticism and that may well cause difficulty in many other cases in which the fields of genetics and physiology are combined.

In Mendelian inheritance it is clear that a single gene may decide whether or not a trait, such as dwarfism in mice, is developed; but to geneticists, at least, it is equally clear that such a gene cannot produce the trait without the cooperation of many other genes. A gene is one factor in a very complex chemical equation. Just as the introduction of the same compound into different chemical equations does not necessarily lead to the same modification of the result, so the effect of a given gene may differ according to the genetic equation into which it is introduced.

That the gene for dwarfism may be modified in its effect by the different gene complexes of different strains of mice appears not to have been considered as a possibility by Boettiger and Osborn (2). They challenge the significance of experimental results with the dwarf gene in one gene complex on the basis of their observations on the dwarf gene combined with a different gene complex. It is, however, not only a possibility but an unquestionable fact that the physiological characteristics of dwarf mice are modified by other genes with which the gene for dwarfism may be associated. The dwarfs raised in the Harvard and Carnegie laboratories are probably due to the same gene. But the Carnegie dwarfs carry the gene complex of the purebred Silver strain brought directly to the MacDowell colony in this laboratory from England by Dr.

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L. C. Dunn in 1927, while the Harvard dwarfs carry an undefined complex (or varying complexes, as suggested by the wide variability between different dwarfs) resulting from hybridization between the Silver and other strains at the Bussey Institution. In one case the dwarfs grow significantly after weaning (growth rate said to equal 'normal' Bar Harbor data); in the other case they do not.

But even if the genetic formulae of the dwarfs were identical it would still be unsafe to use as controls dwarfs raised under different conditions. Aside from possible

TABLE 1. WEIGHT CHANGE IN UNTREATED SILVER DWARFS USED AS CONTROLS FOR ASSAYS OF GROWTH-PROMOTING AND OTHER HORMONE PREPARATIONS

Untreated			Treated[2]			
Sex	Weight at start, gm.	Percentage initial weight,[1] (at end)	Sex	Weight at start, gm.	Percentage initial weight (at end)	Preparation or hormone
♀	7.5	109	♂	7.0	280	Phyone 41B
			♀	8.2	245	Phyone 41B
♀	6.2	105	♂	7.0	192	Phyone 41C
♀	6.7	106	♂	10.0	260	Phyone 44
♂	8.5	103	♂	7.0	172	Collip Q21
♀	6.3	104	♀	7.0	136	Collip Q21
♀	8.2	106	♂	8.5	121	Collip Q16
♂	7.2	107	♀	7.0	136	Collip Q16
♂	6.3	106	♂	6.6	164	Collip PoG
♀	7.8	101	♀	8.6	134	Collip PoG
♀	7.0	98	♂	8.5	172	Lee-Schafer 720
			♀	7.8	154	Lee-Schafer 720
♀	5.5	104	♂	6.6	135	Thyreotropic 243
			♀	7.2	133	Thyreotropic 243
♀	6.5	103	♂	5.7	144	Prolactin 237 heated
			♀	6.0	118	Prolactin 237 heated
♀	6.7	106	♀	8.2	230	Prolactin 237+Thyrotropic 243
			♀	8.0	213	
♀	8.0	104	♀	9.4	128	Prolactin 380, heated
			♀	9.5	147	Prolactin 380, heated
			♂	7.5	184	Desiccated thyroid
			♀	8.0	153	Desiccated thyroid
♀	8.6	102	♀	7.8	146	Desiccated thyroid
			♀	6.4	163	Desiccated thyroid+Prolactin 380
			♀	7.3	146	Desiccated thyroid+Prolactin 380
♀	6.2	97	♀	6.7	93	Prolan-Elberfeld
			♀	7.2	74	Mare serum
♀	6.4	102	♀	8.6	99	Mare serum
♀	7.1	101				

[1] 100×final wt./initial wt.
[2] As previously published.

differences in diet and temperature control, the Harvard practice is to leave the pregnant females in the mating pens where the young are born, nursed and weaned in the presence of the male and other adult females, and nothing is said about limiting the number of young nursed by one female(3). Under these conditions it is not surprising that the Harvard dwarfs show a loss in weight beginning at 17 days. A nursing mother approaching parturition, and other adults stimulating the activity of the young, may well provide an adequate explanation of this loss in weight by the dwarfs. Boettiger and Osborn consider this loss to be the most striking aspect of dwarf growth, meaning of course their dwarfs, under their conditions. The endocrine

deficiencies of Silver dwarfs render them peculiarly sensitive to their surroundings at all ages. Benedict and Lee (4) report the lower limit of the zone of thermal neutrality for these Silver dwarfs as 6° C. higher than for normal laboratory mice. And Boettiger (5) subsequently reported that the loss at weaning could be eliminated by raising the environmental temperature.

When, according to the Carnegie practice, the pregnant mothers are isolated and competition is reduced by limiting the number of young nursed by one female to four, and the natural process of weaning is accomplished in the calm of a box with a non-

TABLE 2. AGE AND BODY WEIGHT OF UNTREATED SILVER DWARF MICE

Age Period, in Days	Number of Mice Weighed	Number of Weight Records	Number of Mice		Body Weight in Grams		
			With 3 or less weight records	With 10 weight records	Individual record		Mean[1]
					Minimum	Maximum	
20–29	20	66	13	0	5.7	10.8	8.1
30–39	56	149	53	0	5.0	10.1	7.2
40–49	58	166	54	3	5.4	9.5	7.2
50–59	44	131	37	6	5.5	9.9	7.4
60–69	30	144	20	9	5.3	9.3	7.2
70–79	28	171	12	13	5.3	8.9	7.1
80–89	26	193	6	15	5.4	9.2	7.1
90–99	24	217	1	19	5.6	8.7	7.2
100–09	28	221	3	17	5.8	8.9	7.3
110–19	29	215	6	15	5.8	9.5	7.4
120–29	30	271	3	21	6.0	9.8	7.5
130–39	30	236	7	19	5.5	10.1	7.5
140–49	21	157	4	13	6.3	8.9	7.4
150–59	16	127	4	11	6.5	8.9	7.5
160–69	16	73	7	3	5.8	9.2	7.6
170–79	10	59	5	4	6.1	8.3	7.1
180–89	11	77	3	4	6.2	8.4	7.3
190–99	11	41	8	1	6.6	8.5	7.5
200–09	7	29	5	1	6.8	8.7	7.7
210–19	8	29	6	1	6.6	8.7	7.8
220–29	6	34	3	3	6.4	8.6	7.5
230–39	5	32	2	2	6.7	8.7	7.4
240–49	4	24	2	1	6.5	9.0	7.3
250–59	3	18	1	1	6.6	8.8	7.9

[1] Average of average weight per mouse per decade.

pregnant mother as the only adult, our dwarfs do not show the loss in weight during weaning reported by Boettiger and Osborn. Such a difference in practice might seem to minimize rather than explain the later differences in growth, but in any case it distorts the measure of the genetic differences between the two kinds of dwarfs and emphasizes the danger of assuming direct comparability of data from different laboratories.

Growth of untreated dwarfs of the Silver strain. Table 1 gives data for the untreated dwarfs held as controls on daily variations due to food and environmental conditions. The data for the treated animals of our previous paper are repeated here to permit a direct comparison with their respective controls. For each group the control was weighed on the same days as the test animal. At the end of the experimental period the changes in the weights of the untreated dwarfs ranged from −3 to +9 per cent of their starting weight. The last three cases in the list of treated mice, given mare serum or prolan-Elberfeld, serve as further (injected) controls. These percentage differences serve for controls on the percentage differences calculated in the same way

for the treated mice, but they do not indicate any growth of the controls, for the weights frequently rise or fall temporarily as much as 0.5 gm. (7 per cent of a 7 gm. mouse) over a period of days or even between successive days.

Since age was found to play so small a part in determining the size of a Silver dwarf after the first 3 weeks no systematic study of the relationship of age to weight has been made; but during the course of time 112 untreated dwarfs have been followed over different periods of time, and weighed at different intervals, for a total of 2880 records. To publish these records in full is not warranted, and any summarization presents difficulties. Table 2 attempts to meet these difficulties by subdividing these data into age decades and by giving the maximum and minimum weight recorded within each decade. To provide some measure of significance we also record the number of mice, the number of weighings and the number of mice with 10 records (that is, weighed daily within the decade) and the number with 3 or fewer records. The last column gives the mean of average weight per mouse for the decade; each mouse has equal influence, although this rests on 1 to 10 readings per mouse. Two mice were weighed at least twice a week beyond 259 days; one weighed 6.7 gm. at 325 days, the other 7.1 gm. at 358 days. The outstanding feature of these figures is their uniformity through 24 decades. Slight changes appear in the course of time, but the shifting population permits little significance to be given to them; the maxima in the second 12 decades are clearly lower than in the first 12, while the reverse is true of the minima. One record of 10.1 gm. at 130 days stands out conspicuously, but this same animal 2 days before and 4 days afterwards weighed 9.6 gm. The high values for the 20 to 29-day decade are, however, unquestionably significant. Rather than a loss during natural weaning, our dwarfs pass this period with ease and may rise to their highest weight within the first month; at this time they are often obviously fat. Removal from the mother at the end of the first month quickly reduces this fat which is rarely regained in later life. The drop in the means from 8.1 to 7.2 gm. is the only reliable change in this column and this is directly related to the removal from the nurse. Obviously purebred Silver dwarfs do not show any of the wide variability of the dwarfs used by Boettiger and Osborn, which at 263 days ranges all the way from 7 to 25 gm. and even 34 gm. at 500 days. These authors are decidedly correct in stating: "Furthermore, attention must be paid to the particular dwarfs used, and an appreciation of the variability normally found must enter into the interpretation of results."

Recombination of anterior pituitary fractions. In our earlier paper a dosage-response line was given, but, on account of the limited number of animals, this included only separate anterior pituitary fractions. However, we were led to assume that the same slope would hold for the combined fractions. This assumption has now been tested.

The two fractions used in this study include prolactin *no.* 680 (11 units per mg.), made by the procedure of Bates and Riddle (6) from cattle pituitaries; this preparation was practically free of adrenotropic action and contained <0.4 per cent as much gonadotropin and <1.0 per cent as much thyrotropin as did the other fraction used. This second (isoelectrically soluble) fraction was *no.* 549. In its preparation from cattle pituitaries the process of solution at the isoelectric point in water, and precipitation with ethanol, was repeated once. When the final ethanol precipitate was dried with acetone it was relatively free of prolactin (none indicated by total doses of 20 to 40 mg. in doves) but contained nearly all of the other anterior pituitary principles (little adrenotropin).

Equal parts by weight of these two fractions were given to different dwarfs in total amounts from 1 to 2000 micrograms, diluted to make doses of 0.05 cc. or 0.10 cc. Intraperitoneal injections were given daily for 30 days. Before starting treatment,

the mice were weighed daily until accustomed to being handled. Subsequent weighings were made once or twice a week, but only the total change in the whole period is shown in figure 1. Each circle represents one mouse. The slope of the line is the same as used in our first paper, namely, for each 10-fold increase in dosage, a 22 per cent increase in body weight is indicated. It is readily seen that as little as 1 μg. daily of the combined fractions caused a 30 per cent increase in body weight of two mice. One microgram is a remarkably small amount of pituitary substance, and it is notable that the growth response of dwarf mice will detect a smaller quantity of combined an-

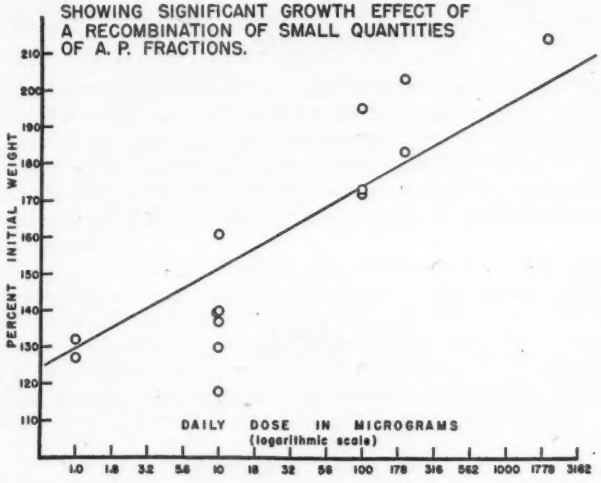

Fig. 1. The increase in body weight of dwarf mice produced in 30 days by combinations of equal parts by weight of two fractions from beef pituitaries.

terior pituitary extracts than will any other gross response except that employed in the local crop-sac test for prolactin. The fit of the curve drawn in figure 1 is as good as might be expected over a dosage range of 2000-fold change in dosage and for as few mice as are available for this extremely interesting type of dwarf. The data appear to warrant our earlier assumption that the same slope holds for the combined fractions as for the separate fractions.

SUMMARY

Physiological studies on the effects of a gene, such as the one for dwarfism in mice, are liable to error from a failure to control the other genes with which the particular one is associated. The untreated Silver dwarf mice held as immediate controls for a previous study fully substantiate the significance of the weight increases reported to result from treatments with prolactin and various anterior pituitary fractions alone and in combination. Further confirmation is provided by all of the weight records of untreated Silver dwarfs that have been gathered in this laboratory, covering ages into the ninth month; after the first month there is little evidence of significant growth.

The hitherto unreported dosage-response curve for combined pituitary fractions is essentially the same as that earlier observed for the separate fractions. Injection for

30 days of as little as 1 μg. daily of two recombined anterior pituitary fractions—one a good prolactin and the other practically free of prolactin—caused a 30 per cent gain in the body weight of Silver dwarfs.

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PITUITARY WEIGHT IN GROWING NEW ZEALAND WHITE RABBITS IN RELATION TO LIVE WEIGHT[1]

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PITUITARY growth relative to normal post-natal body growth in New Zealand white rabbits, although influenced by the functional activity of other endocrine glands (1), exhibits a type of constancy, when large bodies of data are used, sufficient to permit calculation of prediction tables of normal pituitary growth, study of the effect of sex differentiation on the pituitary-body weight relation, and comparison of pituitary size in this breed of rabbit, with that found in other animals of similar body weight.

In other and mixed breeds of rabbits, sex differences in pituitary-body weight relations have not been found significant (2, 3), but in New Zealand white rabbits, the pituitaries of the females have been reported as slightly heavier (4). That breed differences may exist within a species in this respect is shown by the fact that the pituitaries of female albino rats are much heavier than those of the males (5) while a like difference between sexes does not exist in Norway rats. Significantly heavier hypophyses for the female have also been reported for man (6) and dog (7).

EXPERIMENTAL PROCEDURE

The test animals, all New Zealand white rabbits,[2] were fed mixed grain and alfalfa hay, kept at an environmental temperature between 50 and 70°F., and killed during the fall, winter, and early spring months. Neither the males nor the females weighing less than 3000 grams were used for breeding purposes. After each animal was weighed, it was killed by injecting air into an ear vein. The pituitary gland was then removed and weighed to the nearest 0.1 mg. on a precision balance.

ANALYSIS

When plotted on logarithmic paper, the data on paired pituitary and live body weights for 328 male and 370 female New Zealand white rabbits fall along straight lines (fig. 1). Separate equations for male and female of the form $\log Y = \log a + b \log X$ fitted to these data by the method of least squares were transformed to the usual form for relative growth[3] $Y = aX^b$. With pituitary weight, Y, in mg. and live weight, X, in gm., the results are:

$$\text{Male,} \quad Y = 0.265X^{0.58} \tag{1}$$

$$\text{Female,} \quad Y = 0.147X^{0.68} \tag{2}$$

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[2] The authors are indebted for a portion of these data to Messrs. Skat Holst and P. T. Cupps for use of their unpublished data on pituitary weights and live weights for individual rabbits.

[3] For discussions of the relative growth equation with respect to organ size see reference 8,–11.

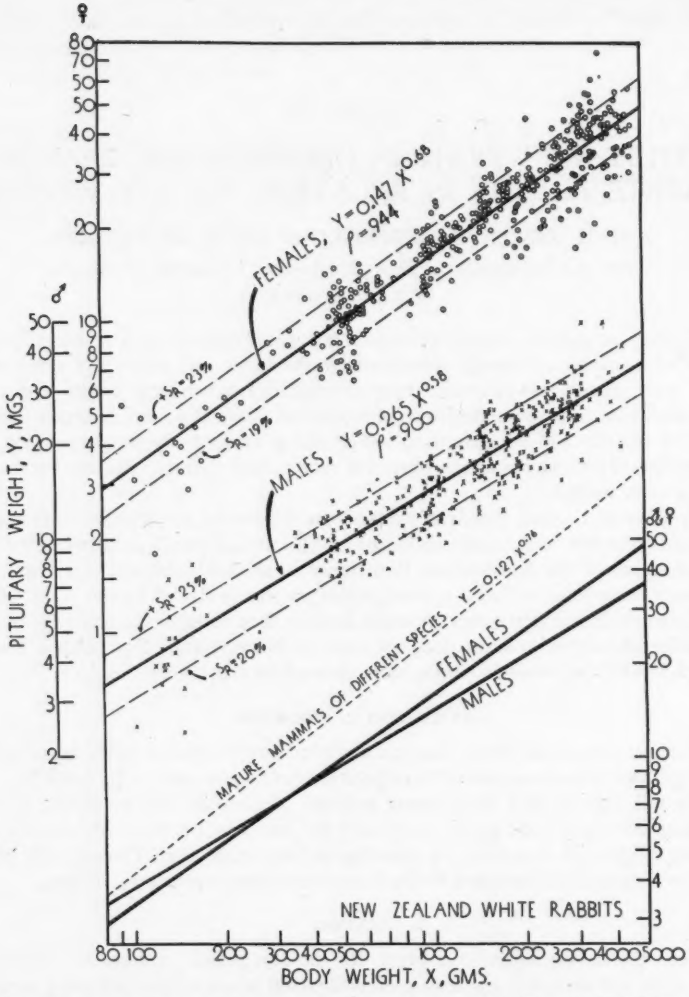

Fig. 1. Relation of pituitary weight to body weight in growing New Zealand white rabbits plotted on a logarithmic grid. The heavy continuous lines represent the equation $Y = aX^b$ fitted by the methods of least squares to the individual data points. The light broken lines on both sides of equation lines represent the standard error of estimate, S_r, including between them in each case two-thirds of the data; the letter rho represents the index of correlation, corresponding to the coefficient of correlation for linear equations; $\pm S_b$, the standard error of the slope indicates the range in values of b that might be expected two of three times in comparable bodies of data for New Zealand white rabbits.

These equations indicate that pituitary weight is not directly proportional to body weight, since exponent b is less than 1. Similar results ($b < 1$) have been reported (cleaned body weights being used) for other breeds of rabbits (2, 3). As exponent b is less than 1, the ratio of pituitary weight to live weight decreases as live weight increases (see table 1).

TABLE 1. PITUITARY WEIGHTS AND RELATIONS IN GROWING NEW ZEALAND WHITE RABBITS DERIVED FROM EQUATIONS (1) AND (2)

Live Weight	Pituitary Weight		Pituitary Weight / Live Weight		Relative Increase[1]		
					In Live Weight	In Pituitary Weight	
	Male	Female	Male	Female		Male	Female
gm.	mg.	mg.	%	%	%	%	%
100	3.83	3.37	.00383	.00337	1	0.579	0.679
200	5.73	5.39	.00286	.00270	5	2.9	3.4
300	7.24	7.11	.00241	.00237	10	5.7	6.7
400	8.56	8.64	.00214	.00216	15	8.4	11.0
500	9.74	10.1	.00195	.00202	20	11.2	13.2
600	10.8	11.4	.00180	.00190	25	13.8	16.4
700	11.8	12.6	.00169	.00180	30	16.4	19.5
800	12.8	13.8	.00160	.00173	35	19.0	22.6
900	13.7	15.0	.00152	.00167	40	21.5	25.7
1000	14.6	16.1	.00146	.00161	45	24.1	28.8
1200	16.2	18.2	.00135	.00152	50	26.5	31.8
1400	17.7	20.3	.00126	.00145	55	29.0	34.7
1600	19.1	22.2	.00119	.00139	60	31.3	37.7
1800	20.5	24.0	.00114	.00133	65	33.7	40.6
2000	21.8	25.8	.00109	.00129	70	36.0	43.4
2500	24.8	30.1	.00099	.00120	75	38.3	46.3
3000	27.5	34.0	.00092	.00113	80	40.6	49.1
3500	30.1	37.8	.00086	.00108	85	42.9	51.9
4000	32.5	41.4	.00081	.00103	90	45.1	54.7
4500	34.8	44.8	.00077	.00100	95	47.3	57.5
5000	37.0	48.2	.00074	.00096	100	49.5	60.2

[1] These relative increases are applicable for interpolation over the entire range of live weights given in the extreme left-hand column. As explained in text, these values may be used for proportioning injections of pituitary and metabolism stimulating materials.

Table 1 also indicates the percentage change in pituitary weight for various percentage changes in body weight. An increase of 100 per cent in live weight corresponds to an increase in pituitary weight of 49.5 per cent in the male, and 60.2 per cent in the female. Similarly, for a 1 per cent increase in body weight, the respective increases in pituitary weight are 0.579 and 0.679 per cent. These latter figures approach very closely the values of the exponents in equations (1) and (2). Exponent b can now be interpreted as the relative change in pituitary weight, Y, corresponding to an exceedingly small unit change in body weight, X.

A priori considerations indicate that the data in table 1 may also be used as a basis for determining hormone dosages affecting the pituitary in growing rabbits of varying

TABLE 2. TEST OF SIGNIFICANCE OF SEX DIFFERENCES IN PITUITARY WEIGHTS IN RABBITS[1]

Source of Variation in Pituitary Weights	Degrees of Freedom	Mean Square Errors of Estimate	Probability that Difference is Due to Chance
Total	696	.0100	
Within sex groups (pooled regression)	695	.0091	
Within sex groups (individual regressions)	694	.0088	
Sex regression coefficients	1	.2550	Less than 1 in 100
Between sex groups (adjusted for body weight differences)	1	.6084	Less than 1 in 100

[1] See reference 12 for table of F ratios and details of method.

live weights. It seems reasonable that such injections should produce effects in proportion to the size of the pituitary, the affected organ. Also, in regard to metabolism, it has been shown that for mature animals of different species, the increase in metabolism with body weight (10) closely parallels the increase in pituitary weight with body weight. On the basis of these considerations, if one male rabbit, for example, is 50 per cent heavier than another, it should be given as a comparable dosage, 26.5 per cent more hormone material than is given the lighter.

That sex differences in relative growth of pituitary weight and body weight are significant is established in table 2 by the application of the methods of covariance. These differences are made evident in the lower part of figure 1, where the male and female fitted curves are brought together on the same scale.

A comparison of the fitted curves for growing rabbits (heavy solid lines, lower part of fig. 1) with the light dotted line for mature mammals of different species (10) indicates that pituitary weight in growing rabbits is at a lower level and increases less rapidly with increasing body weight than is the case in mature animals of different species.

SUMMARY

Individual values of pituitary weight and associated body weights for growing New Zealand white rabbits are presented in a logarithmic chart. An analysis of covariance in pituitary to body weight relations establishes a statistically significant difference due to sex. The ratio of pituitary to body weight is found to decline with increase of body weight during growth. Fitted equations relating pituitary and body weights ($Y = aX^b$) for these growing rabbits are compared with curves for mature animals of different species for a similar range of body weights. Tables for prediction of pituitary weight from body weight and for proportioning pituitary or metabolism stimulating injections according to pituitary weight are presented.

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DETAILED DESCRIPTION OF TECHNIQUE FOR ANDROGEN ASSAY BY THE CHICK COMB METHOD[1]

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CLINICAL INVESTIGATIONS involving assays for androgenic activity have lagged behind those related to the estrogens because of the lack of a convenient and reliable bio-assay technique. Of all of the numerous methods which have been proposed from time to time, that of Gallagher and Koch (1), based on the capon's comb as a quantitative indicator of the potency of injected androgenic material, has been used most extensively. During the past four years we have been engaged in developing a bio-assay method for androgenic activity in urine and tumors, which is relatively simple and of recognizable reliability (for preliminary reports on this work see 2, 3, 4). This investigation has finally been concluded and a detailed report on the work is now nearing completion. Along with a careful description of the procedure, this report will include the evidence, both historical and experimental, which guided us in the development of our method; it will contain also a minute statistical analysis of the data, collected over more than a year on about 1500 chicks, which led us to the final dose-response equation. The mass of experimental and statistical data in such a report, however, obscures the details of assay technique, and the worker who is desirous of using this method without studying the course of its development might lose sight of some of its manipulative details. Accordingly, we are here presenting for the first time, a complete and concise statement of our assay method, and we have attempted to include all pertinent details so that none of these are left to the discretion of the worker who may want to conduct such assays.

PREPARATION OF MATERIAL FOR ASSAY

1. Urine specimens are collected over a 24, 48, or 72-hour period, depending on the purpose of the assay. Storing in a refrigerator is not necessary unless gonadotropic assays are also to be performed.

2. One thousand cc. of the urine is acidified with 100 cc. of 37 per cent hydrochloric acid and at once refluxed for 15 minutes. The flask containing the mixture is then placed in a container of cracked ice to stop further hydrolysis.

3. The hydrolyzed specimen is next extracted with chloroform, in the continuous extractor (5). We have compared this procedure with the benzene method described by Gallagher, Koch, and Dorfman (6), by means of parallel extractions performed on the same specimen of pooled urine. The evidence indicated that, in our hands, the chloroform method resulted in more complete extraction of the sex steroids than the benzene method, and we now use the chloroform procedure exclusively (unpublished data). The arrangement of the apparatus is indicated in figure 1. One-half (550 cc.)

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[1] This investigation was supported in part by grants from the Friedsam Foundation.

of the urine-acid mixture and 200 cc. of chloroform are placed in a one-liter cylinder (*A*), and 500 cc. of chloroform in the two-liter Erlenmeyer flask (*B*) which is provided with a two-holed stopper and rests upon a hot plate (*C*). The flask and the cylinder are supported as indicated in the illustration. A glass siphon tube, with a connecting portion of rubber tubing (*D*), passes from the bottom of the cylinder to the bottom of the flask; the end within the Erlenmeyer flask is provided with a 'J'-shaped outlet as shown. A Hoffman clamp on the rubber portion (*D*) is left open throughout the process; it is closed only after the extraction is completed and during recovery of the chloroform from the sterol residue by distillation, as described below. The glass tube (*E*), which transports the vapor from the flask to the condenser (*F*), has an inner

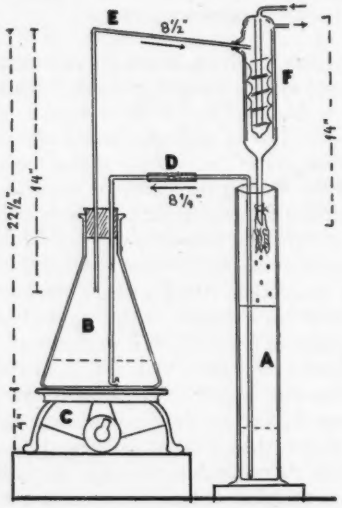

Fig. 1. Apparatus for chloroform extraction of male and female sex hormones from urine.

diameter of 5 to 6 mm. and is covered with asbestos or cotton for thermal insulation. For finer dispersion of the drops of chloroform which pass through the urine, a streamer of glass wool or gauze is attached to the condenser outlet. With this apparatus the condensed vapor is allowed to drip through the urine at the rate of about 35 cc. per minute and is automatically siphoned back to the flask for repeated vaporization. The extraction is continued for 4 hours; investigations now in progress indicate that this time of extraction can be reduced considerably without decreasing its thoroughness, by increasing the rate of chloroform circulation. A battery of 10 such assemblies (2 for each one-liter specimen) is employed for the simultaneous extraction of 5 liters of urine. After completion of the extraction, the tube (*E*) is disconnected from the condenser (*F*), suction is applied to the free end of the tube in order to transfer the chloroform layer from the cylinder to the flask, the screw clamp (*D*) is closed, and the tube (*E*) is re-attached to the condenser. The cylinder containing the urine is then replaced by a receiving flask or bottle and all but about 50 cc. of the chloroform is distilled over. In this way about 90 per cent of the extracting agent is recovered and can be used repeatedly.

4. The chloroform residues in the two flasks are transferred quantitatively to a single evaporating dish and the remaining chloroform driven off under a fan.

5. The residue is taken up in a total of 100 cc. of ether. If estrogen as well as androgen assays are to be made on this material, the two are separated with NaOH at this stage. Otherwise, this separation is not necessary.

6. An aliquot part of this ether solution is transferred to a small Erlenmeyer flask and evaporated at once in an evaporating dish; the residue is preserved for future contingencies. The procedure for estimating the volume of this aliquot fraction based on the estimated activity of the urine specimen, will be described in step 6 of the next section.

7. The residue from the aliquot part is taken up in 10 cc. of sesame oil, and this solution is employed in the assay.

CONDITIONS AND TECHNIQUE OF THE ASSAY PROCEDURE

The following steps are from the detailed report on various statistical considerations entailed in the development of the chick-comb method. In describing a method for chemical analysis or bio-assay, it is of the utmost importance that all of the working conditions be defined explicitly. Likewise, any attempt by other investigators to apply such a method must be performed with rigorous adherence to these conditions; deviations from them must be explicit and justified by adequate experimental evidence (7). And finally, it must be remembered that a statistical evaluation of the reliability of the method is valid only under the working conditions specified.

1. *Maintenance conditions.* The chicks are kept in a brooder (Backwarmer Electric Starting Brooder, Oakes Mfg. Co., Tipton, Ind.) which can accommodate as many as 250 chicks on a floor space of 3.5 by 5 ft. (height, 6 ft.). It consists of five main compartments, each of which is further subdivided into quarters by means of fine wire mesh, thus giving a total of 20 compartments, 18×28″ in area. Each subdivision has its own food tray. Floors of large mesh screening allow droppings to accumulate on an underlying removable tray, beyond reach of the chicks. Temperature is kept between 88° and 96° F. by thermostatic control. On the advice of the Department of Poultry Husbandry, Cornell University, we adopted 'chick-starting mash' (Beacon Mills, Cayuga, New York) as the sole ration; fresh water is given *ad lib.* in troughs. By limiting the number of chicks in any one compartment we avoid overcrowding (which interferes with feeding and favors injury by trampling) and thus keep the mortality at a very low level. Of 5719 chicks used during a period of 3 years, our loss was only 65, or 1.1 per cent. Each compartment of the brooder contains only those chicks (16 to 18 at most) which are being treated with the same solution of androgenic material in sesame oil. Concerning the influence of illumination, we feel that this is probably insignificant, although we have never fully investigated this factor ourselves. Actually, our brooder is kept in a room which gets no direct daylight and only a minimum of artificial illumination during working hours.

2. *Breed of chick.* The single-comb White Leghorn chick is considerably more responsive to the administration of androgen than other varieties; furthermore, this breed of chick is always and everywhere available, and it has satisfied our requirements as to nardiness and uniformity. For these reasons we have adopted the single-comb White Leghorn as a standard for our purposes. Our chicks are obtained from Hall Bros., Wallingford, Conn., and come from a stock tested for pullorum disease by the hatchery.

3. *Age of chick.* All of our chicks are 2 to 3 days of age at start of assay, thus desig-

nated because of the 24-hour span during which the batch of chicks was hatched. Our animals are hatched in the interval from Monday A.M. to Tuesday A.M.; they are shipped to us on Tuesday, arrive Wednesday, and receive their first application on Thursday A.M., when they are 2 to 3 days of age.

4. *Duration of assay.* Since the 'total dose' of assay material is administered as a divided dose rather than in one single application, the duration of assay represents the number of days (seven) on each of which application of assay material is made. Hence, the first application is made when the chicks are 2 to 3 days of age and the last when they are 8 to 9 days of age; they are killed by means of chloroform on the following day, when they are 9 to 10 days old.

5. *Site and mode of administration.* The technique for administering the test substance is simple and utilizes an ordinary 1 cc. tuberculin syringe, fitted with a hypodermic needle. The solution of the test substance is applied by moving the needle lightly over the entire surface of the comb. We are careful to apply the material slowly enough so that spreading to the adjacent head feathers is minimized, and we refrain from inunction in order to avoid possible mechanical stimulation of comb growth or loss of material.

6. *Volume and preparation of oily solution for assaying.* The portion of urine extract which was contained in the aliquot part of the ether solution referred to under 'Preparation of Material for Assay,' (step 6) was dissolved in 10 cc. of sesame oil. Of this material, 0.05 cc. is used for each daily application to each chick; for 16 chicks, treated for 7 days, the total volume required is 16+7+0.05 or 5.6 cc. Thus, the initial preparation of 10 cc. of this solution provides a sufficient excess for all contingencies.

7. *Measurement of comb response.* Comb size is determined by excision and weighing, after the chicks have been killed and their body weights determined. Two parallel longitudinal incisions are made with a sharp (removable blade) scalpel, along the base of the comb at its juncture with the scalp, the boundary being clearly demarcated in 9 to 10-day-old chicks. The incisions extend vertically down to the skull. The entire comb is then removed by undercutting directly on the skull, starting at the posterior extremity of the comb base and moving the scalpel forward to the juncture of the anterior attachment of the comb to the beak. Thus, the removal is accomplished by three simple, clearly defined incisions, each one involving a single continuous movement of the scalpel, to avoid frayed edges. The comb is grasped lightly with a pair of fine forceps, its base is touched lightly to a towel in order to remove blood from the cut surface, and it is transferred immediately to a suitable torsion balance and weighed to the nearest milligram. The amount of blood which has to be removed from the base of the comb before weighing is ordinarily very scant, because of the time (about 10 minutes) which elapses between cessation of circulation and excision of the comb. In order to avoid loss of tissue fluids by squeezing, the comb is grasped only once, with the forceps, during the entire procedure. Minor variations in this technique undoubtedly contribute to the scatter of comb weight data, and for this reason we carefully avoid deviations from our procedure, such as dissection with scissors, desiccation of comb, fixation with formalin, or delayed weighing.

8. *Determination of body weight and sex.* The initial body weight of each chick is measured to the nearest gram on the day of the first application of androgen solution. At the same time, each chick is banded with an individual number for identification, to avoid confusion which might otherwise arise in handling 200 or more birds. The terminal body weight is measured immediately after the chick is killed, following which the comb is excised and the sex determined by laparotomy and

inspection of the gonads. In male chicks, the two bright yellow ellipsoidal testes lie on opposite sides of the vena cava. In females, the single lobulated ovary (grayish-pink to dark crimson in color) overlies this vein; occasionally, a tiny rudimentary second ovary may also be found to the right of the large gonad.

9. *Rejection of data.* A survey of the data on approximately 1500 chicks used in our experiments for developing the dose-response equation revealed that their comb-weight values fell within the range 16 to 165 mg. For the initial body weights the range was 28 to 50 gm., and for the terminal body weights it was 34 to 100 gm. Hence, any chick whose comb or body weight falls outside these several ranges is not suitable for use according to our assay technique, and the data for this individual must be excluded from consideration in calculating the results of the assay.[2]

CALCULATION OF THE FINAL RESULT OF ASSAY

For practical purposes, and in order to simplify the process of calculation, we have developed a work chart (table 1) which covers all steps in the assay. By using this the calculations are reduced to a matter of simple arithmetic, without any need for employing the dose-response equation as such. A description of this chart and an illustration of its use are given on page 69.

From the data obtained in the foregoing manner, the sum of all of the comb weights is calculated for all 16 chicks of the same group, independent of sex, as well as the sums of all the initial and all of the final body weights. In addition, each comb weight is squared and the sum of these squares is also calculated. The resulting totals are then substituted in the following dose-response equation:

$A/ch =$

$$\frac{1.061(\Sigma W) - 0.0043(\Sigma W^2) - 0.397(\Sigma B_i) - 0.267(\Sigma B_t) + 14.75\mathcal{N}_m + 18.54\mathcal{N}_f}{100(\mathcal{N}_m + \mathcal{N}_f)} \quad (1)$$

wherein the symbols have the following meanings:

ΣW = the sum of the comb weights, expressed in mg.;
ΣW^2 = the sum of the squared comb weights;
ΣB_1 = the sum of the initial body weight, expressed in gm.;
ΣB_t = the sum of the terminal body weights, expressed in gm.;
$\mathcal{N}_m$ = the number of male chicks used in the assay;
$\mathcal{N}_f$ = the number of female chicks used in the assay;
A/ch = the androgen activity fo that portion of the original specimen which was applied to a *single chick*, expressed in decimilligrams (1 dmg. = 100 micrograms).

As a simple measure of the androgen activity of urine or other material, we propose the expression 'androsterone equivalents' (And.-eq.) which is defined as the number of decimilligrams (dmg.) of crystalline androsterone[3] which yield the same adjusted comb growth response as the quantity of urine or other extract employed.

[2] It is worthy of note that, of 809 chicks used for assays of known quantities of crystalline androsterone (see the following article by Klempner, Hollander, Frank and Kriss), one had an initial body weight slightly above the standard range (54 gm.) and two had comb weights one mg. below (15 mg. each).

[3] We have used androsterone in preference to other androgenic substances such as testosterone or testosterone propionate because *a*), the international standard of male sex hormone activity is based upon this substance, and *b*), from the clinical point of view, androsterone is the chief comb growth-stimulating substance excreted in human urine, whereas the other biologically active androgens mentioned above have not been recovered from this excretion.

The decimilligram is used as a measure of weight because it corresponds to the international unit of androgenic activity. Hence, the value *A/ch* obtained from the dose-response equation (*column 20* of the chart) is expressed as 'And.-eq. per chick.' In order to convert this to 'And.-eq. per liter of urine' we must divide it by the volume of urine equivalent to the total amount of urine extract which was used for each chick (*column 21* of the chart). Thus, if we let

$A/1$ = the androgen activity of the specimen, expressed as And.-eq. per liter;
U = the total volume (in liters) of urine extracted (usually 1 liter);
E = the total volume (in cc.) of ether used for dissolving the extract from U liters of urine (ordinarily 100 cc.);
V = the aliquot portion of this ether solution (in cc.) taken for preparation of the solution in sesame oil;
So = the total volume of oil (in cc.) used to dissolve the residue from V cc. of ether,

then

$$A/1 = (A/ch) \div \left[\frac{U \times V \times 0.35}{E \times So} \right] \tag{2}$$

where 0.35 is the total number of cc. of oily solution used on each chick.

If the androgenic activity of a urine extract be wanted on a *per diem* basis, we use the formula

$$\text{And.-eq./day} = (\text{And.-eq./liter})(\text{number of liters of urine excreted per day}). \tag{3}$$

If the assay is performed on tissue extract instead of urine, then the activity per gram of tissue can be calculated by means of *equation 2*, provided U is taken to represent the number of grams of tissue originally extracted.

An explanation of our routine procedure for determining the volume of the ether solution which is to be taken for preparation of the oily solution (V in *equation 2*) may save the user of this method considerable time and confusion. Before starting an assay, we make a provisional estimate of the activity on the basis of our past experience with similar cases; thus, we use 10 And.-eq./liter of urine for normal adult females, 30 for normal adult males, 5 for normal pre-puberal individuals of either sex, and 100 for adrenal cortical carcinoma. If such a preliminary estimate is impossible, we make one of more trial assays at different estimated levels of activity (e.g., 10, 30, or 60 And.-eq.) using only 5 chicks for any one trial. For such a trial assay, all of the foregoing equations are applicable. However, because a smaller number of chicks is used, the residue from an aliquot portion of the ether solution is dissolved in only 2.5 cc. of sesame oil, instead of 10 cc. (So in *equation 2*), thus saving some of the active material; note of this is taken in the routine manner for the calculation of $A/1$ from A/ch. Then, having made a preliminary estimate of the androgen activity, by either of these methods, we calculate V to be used in the definitive assay by *equation f*.

$$V = \frac{E \times So}{U \times 0.35} \times \frac{(A/ch)}{(A/1)_{est}} = \frac{100 \times 10 \times 0.25}{1 \times 0.35 \times (A/1)_{est}} = \frac{711}{(A/1)_{est}} \tag{4}$$

in which

E = 100 cc. (ether),
So = 10 cc. (oil),
U = 1 liter (urine),
$(A/1)_{est}$ = the preliminary estimate of activity per liter,
(A/ch) = 0.25 And.-eq.

The value for (A/ch) is set at 0.25 And.-eq. (i.e., 25 μg.) for the following reason. The dose-response equation is applicable in a dosage range (i.e., A/ch) of 0 to 0.50 dmg. (0–50μg.). However, a study of the distribution of expected errors throughout the allowable dosage range indicates that there is a marked tendency for values of A/ch to be too large below a middle position in the neighborhood of 0.25 And.-eq. and to be too small above this middle value. Hence, an assay which yields an A/ch-value in the neighborhood of 0.25, because of a judicious choice of V, is subject to less experimental error than is an assay at any other level of A/ch.

The sheet for the final calculation of A/ch and A/l from the observations, without any thought about the algebra entailed, is given in table 1. In order to show how

TABLE 1. ANDROGEN BIO-ASSAY CHART[1]

Assay Number	Subject	Preparation of Urine for Assay			
		Volume of original specimen (liters)	Volume of urine hydrolyzed and extracted (liters)	Fraction of extract per chick $\frac{\text{cc. ether used}}{\text{total cc. ether}} \times \frac{\text{cc. oil/chick}}{\text{total cc. oil}}$	Volume used per chick, (liters) (4)×(5)
(1)	(2)	(3)	U (4)	$(V/E)\times(0.35/So)$ (5)	(6)
42G	E	1.28 (2 days)	1.0	10/100×0.35/10=0.0035	0.0035

Summary of Assay					
Sum of comb weights	Sum of squared comb weights	Sum of initial body weights	Sum of terminal body weights	Number of chicks	
				Male	Female
ΣW (7)	ΣW^2 (8)	ΣB_i (9)	ΣB_t (10)	N_m (11)	N_f (12)
1520	146,560	625	1144	9	8

Calculations							Calculated Androgen-Equivalents	
+1.061 × ΣW	−0.0043 × ΣW^2	−0.397 × ΣB_i	−0.267 × ΣB_t	+14.75 × N_m	+18.54 × N_f	Algebraic sum of (13) through (18)	Per chick $\frac{.01\times(19)}{(11)+(12)}$ (dmg./ch.)	Per liter $\frac{(20)}{(6)}$ (dmg./l.)
(13)	(14)	(15)	(16)	(17)	(18)	(19)	A/ch (20)	A/l (21)
1612.72	−630.21	−248.13	−305.45	132.75	148.32	710.00	41.76	119.31

[1] The three sections of the chart should be read as a continuous strip. Numbers of columns are given in parentheses.

it is used, we present an illustrative assay, as follows: The patient was a 19-year-old female with the history, physical examination, and laboratory findings typical of an adrenal cortex carcinoma, proved subsequently at operation. A 48-hour pre-operative specimen of urine, with a volume of 1280 cc. (*column* 3 of the table), was collected for assay, and one liter of this (*column* 4) was hydrolyzed and extracted. Our provisional estimate of the activity $(A/l)_{est}$ in this case was 70 And.-eq., about 7 times the

value for a normal female; substituting this in *equation 4* we obtained a value of 10 (actually 10.2) for V, the aliquot volume of ether solution to be used. Since $E = 100$ and $So = 10$, we substituted these values in *column 5* and got 0.0035 for the fraction of the total quantity of extract which was used per chick. This fraction corresponds to 0.0035 liter of urine per chick, *column 6*. The assay was performed in the usual way except that we started with 18 chicks, one of which died in the course of treatment. Of the 17 survivors, 9 were males (*column 11*) and 8 were females (*column 12*). The sum of all 17 comb weights (ΣW) was 1520 mg. (*column 7*), and when each comb weight was squared their sum (ΣW^2) amounted to 146,560 (*column 8*). The sum of the 17 initial body weights (ΣB_i) was 625 gm. (*column 9*) and the sum of their terminal body weights (ΣB_t) was 1144 gm. (*column 10*). This completed the summary of the assay data. The calculations were then made without direct reference to the assay equation, as indicated in *column 13* through *18*; e.g., the sum of the comb weights from *column 7* was multiplied by its factor (+1.061) and the product (1612.72) entered in *column 13*, and so on for the remaining columns. The algebraic sum of all 6 products in *column 13* through *18* (710.00) was entered in *column 19*, and when this was divided by the total number of chicks (17) from *column 11* and *12* (and multiplied by 0.01 to convert μg. to dmg.) we obtained a value of 41.8 dmg. for A/*ch* (*column 20*). Finally, we divided this value by 0.0035, the volume of urine used per chick (*column 6*) and obtained 119 dmg./liter as the androgen activity of the original specimen. To convert this to a *per diem* basis, we may multiply it by the average volume or urine collected per day (1.28/2 = 0.64 liter); the resulting product is 76 dmg./day.

Our technique, as described, involves many detailed steps, yet it is not difficult of performance. It may be applied to material obtained by any adequate method of extraction. Variations involving different vehicles, quantities and number of applications will nullify the applicability of our dose-response equation. We therefore emphasize that the details of our technique must be adhered to rigorously if any valid comparison of data is contemplated.

We wish to express our thanks to Dr. Erwin Schwenk of the Schering Corp., Bloomfield, N. J., for supplying the androsterone used in this work.

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REPORT ON ASSAYS OF KNOWN QUANTITIES OF ANDROSTERONE BY THE CHICK METHOD[1]

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THE PRECEDING PAPER (1) contains a detailed description of our technique for the bioassay of androgens by means of the comb growth reaction in the baby chick. In that publication, it was emphasized that the applicability of the method is limited to the dosage range of 10 to 50 micrograms of androsterone, such low concentrations being attained by dissolving the crystalline material in a suitable volume of sesame oil. From the statistical analysis of the data, however, it was anticipated that greater accuracy would be obtained within the range 20 to 40 μg than outside it. In order to test the reliability of our method, we have performed a series of 39 determinations with known quantities of crystalline androsterone, in the usual way, using a group of 16 to 18 chicks for each unknown. None of these data was included in the 10 series of experiments on which the dose-response equation was based. The assays here reported were performed over a period of 3 years, without any selection of month, and they employed 19 different consignments of chicks. Consequently, variations due to season, differences ascribable to variation of batch, and similar factors are all covered by this study.

The results of this experiment are presented in table 1. For the 24 determinations in the dosage range of 20 to 40 μg., the mean error is 4.1 μg. or 13.0 per cent, whereas all 39 determinations in the entire dosage range of 10 to 50 μg. possess a mean error of 5.7 μg. or 24.6 per cent. It will be observed from the table that the errors tend to be minimal for dosages around 30 μg.; below this central value they tend to be positive and above it they tend to be negative. This is consistent with our expectation, as presented in the foregoing paper. A statistical analysis of these errors will be published later.

It is worthy of note that three of these assays (*16 B*, *C*, and *D*) were performed with a batch of chicks that had been exposed to severe cold while in transit. Many chicks of the original shipment died before or shortly after arrival and the survivors all showed characteristically low body weights and comb growth responses. In spite of this, the errors for these three assays are entirely within the over-all range for the other determinations. This illustrates the efficacy of the system of body-weight corrections employed in our dose-response equation.

These results give evidence of the practical applicability of the method. They likewise demonstrate that, in the assay of an unknown, it is advisable to perform one or two preliminary assays, using 4 or 5 chicks for each. From these trials, a close estimate can be obtained of that concentration of extract in sesame oil which is required

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TABLE 1. ASSAYS OF KNOWN QUANTITIES OF ANDROSTERONE BY CHICK METHOD

Assay Number	Expected Value (μg./chick)	Value Obtained (μg./chick)	Error	
			(μg./chick)	(%)
29B	9.8	16.2	+ 6.4	64
28B	9.8	14.1	+ 4.3	43
36J	10.0	17.2	+ 7.2	72
37A	10.0	16.1	+ 6.1	61
16B	10.0	16.2	+ 6.2	62
40G	10.0	15.2	+ 5.2	52
26E	12.0	18.1	+ 6.1	51
28C	14.7	19.5	+ 4.8	36
29C	14.7	18.9	+ 4.2	31
26F	15.0	20.0	+ 5.0	33
37B	19.9	20.6	+ 0.7	4
35I	20.0	21.6	+ 1.6	8
39C	20.0	28.4	+ 8.4	42
16C	25.0	27.8	+ 2.8	11
46I	26.3	27.1	+ 0.8	3
48L	26.3	24.1	− 2.2	8
49K	26.3	24.7	− 1.6	6
44I	28.0	30.0	+ 2.0	7
38I	29.9	26.6	− 3.3	10
37C	29.9	25.2	− 4.7	16
34G	30.0	26.1	− 3.9	13
36K	30.0	25.4	− 4.6	15
39E	30.0	31.7	+ 1.7	6
40D	30.0	29.2	− 0.8	3
42E	30.3	32.8	+ 2.5	8
42B	30.3	29.1	− 1.2	4
43J	35.0	27.8	− 7.2	21
43H	35.0	31.3	− 3.7	11
45K	35.0	36.0	+ 1.0	3
45J	35.0	26.9	− 8.1	23
47K	35.0	31.0	− 4.0	11
37D	39.9	27.9	−12.0	30
35J	40.0	27.4	−12.6	32
39G	40.0	33.4	− 6.6	17
46J	43.8	32.9	−10.9	25
37E	49.9	32.9	−17.0	34
16D	50.0	36.7	−13.3	27
39I	50.0	35.8	−14.2	28
40C	50.2	35.7	−14.5	29

for a dosage close to 30 micrograms per chick. This concentration can then be used for the definitive assay using 16 chicks.

We wish to take this opportunity to express our thanks to Dr. Erwin Schwenk of the Schering Corp., Bloomfield, N. J., for supplying the androsterone used in this investigation.

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EFFECT OF RAT'S LIVER ON ACTIVITY OF TESTOSTERONE AND METHYL TESTOSTERONE[1]

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IT IS KNOWN that many steroid hormones are less potent orally than parenterally. Methyl testosterone is the most efficient androgen by the oral route because it loses less of its parenteral effectiveness than does either testosterone or testosterone propionate.

There is evidence that the endogenous testicular androgen of the rat is inactivated by the liver (1, 2). If orally administered androgens are absorbed from the digestive tract into the portal blood stream, they would necessarily pass directly to the liver and, if the liver also inactivates these androgens before they pass into the general circulation, their relative ineffectiveness under these circumstances would be understandable.

Testosterone propionate, when absorbed directly into the portal blood stream from pellets located in the spleen, is ineffective in the castrated rat (3, 4). This finding is consistent with the idea that liver inactivation is responsible for the diminished oral effectiveness of testosterone propionate. However, the finding that methyl testosterone is also inactivated by the liver (5) precludes the converse assumption that methyl testosterone is relatively more active orally because it is not inactivated by the liver. It was thought possible that methyl testosterone might be inactivated by the liver to a lesser degree than other androgens, thus accounting for its greater oral efficiency. The following experiments were devised to test this possibility.

PROCEDURE AND RESULTS

Pellets of pure crystalline testosterone and methyl testosterone were implanted into the mesentery of the small bowel (technic previously described in detail, 6) of castrated male rats, 100 ± 10 gm. in weight. Castration and implantation of the pellets were carried out in a single operation. Eleven days later the animals were killed. At autopsy the ventral prostates and seminal vesicles were removed and weighed and the pellets were recovered after careful search for adhesions to the pellet site. The few animals in which such adhesions were found were excluded from the results. The recovered pellets were cleaned of encapsulating tissues when necessary, although generally they were not covered by tissue other than the mesentery. After 24 hours in a drying oven (at 50°C.) they were weighed on a balance sensitive to 0.02 mg.

Many difficulties are encountered in a quantitative study of hormone effects when pellets are used as the source of the hormone. The initial pellet weights of the hormones compared must be adjusted so that the average daily absorption from each will be equivalent. This involves allowance for differences in molecular weights as well a

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for different rates of absorption. The unpredictable mortality of the experimental animals interferes with this adjustment inasmuch as the average pellet weight for a group, perfectly adjusted at the start, is altered according to which animals die. Methyl testosterone is more slowly absorbed than testosterone and also has a greater molecular weight because of the androgenically inactive added methyl group, therefore slightly larger pellets of this substance were used.

Intramesenteric pellets. In the first experiment (*groups* 1, *a* and 1, *b*, table 1) the anticipated relative daily absorptions from the intramesenteric testosterone and

TABLE 1. EFFECTIVENESS OF PELLET IMPLANTS IN RATS

Experimental Group Number	Duration of Experiment, days	Number of Animals	Average Body Weight, gm.	Substance	Average Initial Pellet Weight, mg.	Average Daily Absorption, mg.	Average Prostate Weight, mg.	Average Seminal Vesicle Weight, mg.
Castrate Controls	11	11	127				14.6	20.9
Intramesenteric pellets								
1, a		4	126	Testosterone	10.28	0.476	99.3	215.8
1, b		5	124	Methyl Testosterone	11.07	0.296	76.0	144.2
2, a	11	4	121	Testosterone	5.19	0.240	51.3	146.3
2, b		4	118	Methyl Testosterone	5.47	0.146	86.6	62.6
3, a	11	10	123	Testosterone	1.01	0.079	56.7	92.5
3, b	11	7	108	Methyl Testosterone	1.56	0.077	81.9	104.7
4, a	11	8	116	Testosterone	0.80	0.049	39.3	53.0
4, b		7	115	Methyl Testosterone	1.07	0.066	56.9	50.9
5, a	21	6	143	Testosterone	2.01	0.083	55.5	101.5
5, b		6	137	Methyl Testosterone	3.16	0.104	83.2	177.2
Spleen pellets								
6, a	11	10	113	Testosterone	0.98	0.053	17.7	20.6
6, b		10	113	Methyl Testosterone	1.24	0.055	45.1	23.2
Subcutaneous pellets								
7, a	11	7	138	Testosterone	1.02	0.057	176.6	365.1
7, b		9	134	Methyl Testosterone	1.24	0.041	161.6	377.6

methyl testosterone pellets were not realized. The daily amount of testosterone absorbed was considerably greater than that of methyl testosterone (0.476 and 0.296 mg., respectively). The prostate and seminal vesicle weights in both groups were considerably above castrate level, indicating that both hormones had been effective. This finding, in view of the relatively large amounts of hormones involved did not appear unreasonable. The liver presumably could not cope with all of the hormone passing through it, and the relative capacity of the liver to inactivate the two androgens could not be judged. Another experimental group was started using smaller pellets.

In experimental *group* 2, *a* and *b* (table 1) the average daily absorption was less than in the first experimental groups, but the testosterone was still absorbed in greater quantity than the methyl testosterone (0.240 and 0.146 mg., respectively). The pro-

state and seminal vesicle weights were again above castrate level with both substances, indicating some effectiveness for the absorbed hormones.

In the third trial (*group 3, a* and *b*) the daily absorption was reduced still further and was approximately equal for testosterone and methyl testosterone (0.079 and 0.077 mg., respectively). Again the prostate and seminal vesicles gave evidence of stimulation. It is interesting to note, in view of further developments, that the daily amounts of hormone absorbed were of the same magnitude as those used in experiments involving pellet implantation in the spleen in which Biskind and Mark (3, 4) with testosterone propionate and Biskind (5) with methyl testosterone, obtained no effect on the castrate accessories.

In *group 4, a* and *b* the daily absorption was still less. Even with these small daily amounts, there was some increase in accessory weights, although only methyl testosterone produced significant increments (critical ratios of 4.27 and 4.58 for prostate and seminal vesicle respectively).

Absorption from pellets may be greater for a few days after implantation than later when the pellet has decreased in size. The possibility arose that the effects obtained in the previous experiments may have been due to a 'flooding' of the liver with amounts greater than it could adequately handle. It was thought that in a longer period of time the absorption rate might decrease to a point at which a difference in the effect of the liver on the two hormones might become appreciable. In the next experiment (*group 5, a* and *b*) the pellets of testosterone and methyl testosterone were left in situ for 21 days. In all other respects the procedure was the same as in previous experiments. The accessory weights in these 21-day groups were comparable to those of the 11-day groups having approximately the same average daily absorption (*group 3, a* and *b*) which indicates that time, within the limits represented, is not an important factor in producing the results. The prime factor is the average daily amount of hormone absorbed. In all of the groups with intramesenteric implants the magnitude of effect was roughly proportional to the absorption.

Intrasplenic pellets. After deliberation on the above results it was considered possible that a part of the hormone absorbed from the intramesenteric pellets might have entered directly into the lymphatics which are very numerous in the intestinal mesentery, thus secondarily arriving in the general circulation without passing through the liver. To test this possibility it was decided to implant pellets into a situation where the lymphatic drainage is less extensive than in the intestinal mesentery. The spleen was selected inasmuch as its lymphatic drainage is limited and its vascular drainage is entirely portal. Pellets of testosterone and methyl testosterone of approximately the same size as in experimental *group 4* were implanted into the spleen (*group 6, a* and *b*). Daily absorptions in these groups were close to those obtained in *experiment 4* and were in the correct ratio for testosterone and methyl testosterone (0.053 and 0.055 mg. respectively).

The testosterone in this experiment produced no significant effect on the accessories while methyl testosterone had a significant effect only on the prostate. Comparing the mean prostate weights with the castrate values, the critical ratios obtained were 1.67 for testosterone and 7.88 for methyl testosterone, indicating a definitely significant effect on prostate weight with methyl testosterone. The seminal vesicles obviously were not significantly enlarged even with methyl testosterone. This difference between the prostate and seminal vesicle is what should be expected when small amounts of androgen are concerned. Moore and Gallagher (7), Moore and Price (8), Burkhart (9), and, in our own laboratory, Nelson and Greene (10) have demonstrated that the rat prostate has a lower threshold of response to androgens than the seminal vesicle and therefore responds to dosages which do not affect the seminal vesicle.

Subcutaneous pellets. As a control procedure, pellets of the size used in *groups 4* and *6* were implanted subcutaneously into castrates of the same size as were used in all the other experiments (*group 7, a* and *b*). The resulting daily absorption of testosterone was approximately the same as in the other groups (0.057 mg.) while that of methyl testosterone was somewhat lower (0.041 mg.). The effects of both substances on the accessory weights were many times greater than those obtained from the intramesenteric or intrasplenic pellets. They were actually greater than the effects obtained from intramesenteric pellets approximately 10 times larger (*group, 1 a,* and *b*).

DISCUSSION

Obviously the pellets of testosterone and methyl testosterone, when located in the mesentery or in the spleen are not as effective as pellets of the same size located subcutaneously. Since in both of the former locations at least some of the absorbed hormone passes directly to the liver through the portal blood stream, it may be concluded that the liver is responsible for the demonstrated loss in effectiveness. The consistent appearance of some effect from both testosterone and methyl testosterone pellets in all groups in which they are located in the mesentery demonstrates that some of the absorbed androgens have escaped liver inactivation. Since testosterone, when absorbed from pellets in the spleen, absolutely failed to produce an effect, it may be assumed that the major part of the absorbed hormone must have passed through the portal blood stream into the liver, where it was completely inactivated. The same amount of testosterone, however, when absorbed from pellets in the mesentery was capable of producing some effect. It is therefore believed that at least part of the hormone absorbed from the intramesenteric pellets probably was picked up by the lymph stream.

Methyl testosterone, in the amount absorbed from the spleen pellets, although directly transported to the liver by the portal blood stream evidently escaped complete inactivation, judging by its effect on the prostate. Some inactivation by the liver must have occurred, however, since this effect was considerably less than the effect produced by a smaller amount of methyl testosterone absorbed from subcutaneous pellets.

The results of these experiments and the consequent conclusion differ from the results and conclusions offered by Biskind. He found that methyl testosterone, when absorbed from pellets in the normally located spleen, had no effect on the castrate accessories, although the average daily absorption in his experiments was greater than ours (0.097 mg.). Several differences in procedure may explain this discrepancy. Biskind inserted the pellets 3 weeks after castration, whereas we inserted them at the time of castration. He was therefore concerned with restitution of the accessories from a previously reached castrate level, while we were concerned with preventing castration atrophy. It is conceivable that the small amount of methyl testosterone which may have escaped liver inactivation in his animals was insufficient to stimulate the already atrophied accessories, while the smaller amount in our animals may have been adequate to prevent complete regression. Since his experimental animals were considerably larger than ours, the difference in quantity of hormone per unit of body weight might also account for the difference in results. Biskind allowed the spleen pellets to remain in situ for 4 weeks whereas ours were in place only 11 days. While the average daily absorption from the pellets in his experiments was greater than in ours, there is a slight possibility that the actual daily absorption during the last part of the 4-week period might have been so small that it was incapable of maintaining the slight effect on the accessories demonstrated in our short time experiments.

Although the primary motivation for the present experiments was the search for an explanation of the relatively greater oral activity of methyl testosterone, it cannot be stated that this explanation has been definitely discovered. Biskind has advanced the hypothesis that the greater oral activity of methyl testosterone may be due to absorption of this substance from the gastrointestinal tract by a route other than the portal circulation, possibly the lymphatic system, thus by-passing the liver. In our intramesenteric-pellet experiments it was thought that some of the hormone may have been shunted around the liver through the lymph stream. If true, this happened to testosterone as well as to methyl testosterone and it could not therefore be considered an adequate explanation for the greater oral activity of methyl testosterone. However, absorption of hormone from a pellet located in the mesentery of the intestine does not duplicate the conditions of absorption from the intestine when the hormone has been administered orally.

From the experimental results reported it is obvious that, given an equal opportunity to inactivate testosterone and methyl testosterone, the liver more efficiently disposes of the testosterone. If, on oral administration, any or all of these two hormones pass into the liver through the portal system, it is possible that a part of the methylated androgen may escape inactivation. If a sufficient dose is given orally, enough may filter through the liver to be effective when it reaches the end organs. The disparity between oral and parenteral effectiveness of methyl testosterone is considerable. Emmens and Parkes (11) have stated that the oral activity of methyl testosterone is only two-fifths of its parenteral activity in the rat. With testosterone, however, they found the oral activity to be less than one-tenth of its parenteral activity.

SUMMARY

The ability of the rat's liver to inactivate testosterone and methyl testosterone was tested by implanting into various locations pellets of both hormones in such sizes that equivalent amounts were absorbed daily. The weights of the accessories compared to the castrate level were used as the criteria for effectiveness of the androgens. Both testosterone and methyl testosterone were markedly effective when absorbed directly into the peripheral blood stream from subcutaneously located pellets. When the pellets were located in the spleen so that the androgens were carried by the portal blood stream directly to the liver, methyl testosterone had some effect but testosterone was completely ineffective. It is concluded from these results that testosterone is more efficiently inactivated by the liver than methyl testosterone. The moderate effectiveness of both hormones when absorbed from the intramesenterically located pellets has been tentatively explained on the basis of at least partial absorption into the lymph stream, by which route both hormones get into the general circulation without first passing through the liver. The results are discussed in relation to the relative oral efficiency of the two hormones.

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EXPERIMENTS CONCERNING SUPPOSED INFLUENCE OF COCK'S HEAD APPENDAGES ON SIZE OF TESTES

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IT IS A WELL-KNOWN FACT that growth of the cock's head appendages, comb and wattles, is under control of the testicular incretion. It was unexpected, however, when Buckner, Insko and Martin (1) and Zawadowsky (2, 3) independently reported that a relationship of opposite nature also exists, that is, that growth of the testes is influenced by removal of comb and wattles. Such a reciprocal effect, if verified, would be of considerable theoretical, perhaps even of applied, interest. It is not

TABLE 1. EXPERIMENTS WITH WHITE LEGHORN MALES AS CONDUCTED BY VARIOUS AUTHORS

Author	Appendages	Age at Operation	Time from Operation to Killing	Control Series					Test Series				
				Number of Animals	Body weight		Testes weight		Number of Animals	Body weight		Testes weight	
					Range, gm.	Mean, gm.	Range, gm.	Mean, gm.		Range, gm.	Mean, gm.	Range, gm.	Mean, gm.
Buckner, Insko and Martin	comb	4 weeks	10 weeks	6	1067–1260	1152	0.8–5.2	2.8	5[1]	1110–1230	1156	5.0–9.0	6.5
									5[2]	900–1350	1117	0.8–8.4	4.3
Zawadowsky	comb and wattles	adult	1 month	13	1240–1830	1513	2–20	7.8	11	1160–1800	1477	5–27	17
Zawadowsky	comb	adult	4 months	10	1500–3100	1804	0.30–4.49	1.84	10	1520–2180	1847	0.52–7.35	2.91
Hoskins and Koch	comb	1 month	13 weeks	5	?	898[3]	1.83–15.54	6.54	5	?	748[3]	0.55–9.54	2.97
Hoskins and Koch	comb	1 month	7 months	13	?	1473[3]	0.9–8.6	3.14	12	?	1553[3]	1.0–21.8	11.04
Marlow and Payne	comb and wattles	8 weeks	4½ months	14	1480–1930	1684.5	0.92–19.54	6.67	10	1800–2100	1941	5.90–25.30	12.97

[1] combs erect at time of removal
[2] combs drooping at time of removal
[3] these figures are not actual weights, but increase in weight between time of operation and killing

surprising, therefore, that several investigators set out to check the correctness of these results. The data, as they exist in the literature to date, are briefly summarized in table 1.

Buckner, Insko and Martin (1) removed the comb from cockerels at the age of 4 weeks and killed the animals when they were 10 weeks old. Zawadowsky (2, 3) used adult cocks; in one experiment he cut off the comb as well as the wattles and determined testes weight 1 month later; in another experiment he removed only the comb and killed the animals 4 months afterwards. Hoskins and Koch (4) made five dif-

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ferent tests in which the ages at removal of the comb were 2 months, 1 month, 1 month, 2 weeks and 1 month, respectively, and the corresponding intervals between operation and termination of the experiment were 44 days, 7 weeks, 13 weeks, 30 days and 7 months. The data of but two of these five experiments are reproduced in table 1. Positive results, i.e., an increased testes weight, were reported only for the experiment in which the comb had been removed at the age of 1 month and the cockerels were killed 7 months later. Marlow and Payne (5) cut off the comb and wattles at the age of 8 weeks and killed the birds after an interval of 4.5 months.

All of these authors claimed that under certain conditions removal of the comb or of both comb and wattles produced, at some later date, increased testicular weight as compared with unoperated control animals. Nikolaiczuk and Maw (6) arrived at the same conclusion; but their results have not as yet been published in detail.

EXPERIMENTAL PROCEDURE

Our experiments on this problem were started in 1937. Several tests have been made since then, but the present report will be based chiefly on experiments which

TABLE 2. MEANS AND STANDARD ERRORS OF BODY WEIGHT AND OF ABSOLUTE AND RELATIVE TESTES WEIGHT OF WHITE LEGHORN MALES

Group	I	II	III	IV	V	VI	VII
Number of birds	48	46	48	45	53	44	52
Body weight, gm.	2154.6±34.2	2244.8±25.8	2232.5±29.2	2193.6±32.3	2230.0±38.4	2183.9±39.8	2153.8±31.5
Testes weight, gm.	8.98±0.78	11.08±0.67	14.32±0.93	9.60±0.74	9.85±0.69	8.30±0.84	7.13±0.68
Testes weight×100 / body weight	0.414±0.0337	0.495±0.0294	0.603±0.0428	0.430±0.0308	0.444±0.0299	0.382±0.0391	0.328±0.0304

TABLE 3. DIFFERENCES IN ACTUAL AND RELATIVE WEIGHT OF TESTES IN WHITE LEGHORN MALES

Difference Between Group Means	Actual Testes Weights		Relative Testes Weights	
	Difference, grams	Difference / S.E.	Difference, grams	Difference / S.E.
I–VII	1.85±1.03	1.80	0.086±0.045	1.91
II–VII	3.95±0.95	4.16	0.167±0.042	3.98
III–VII	7.19±1.15	6.25	0.275±0.053	5.19
IV–VII	2.47±1.00	2.47	0.102±0.044	2.32
V–VII	2.72±0.97	2.80	0.116±0.043	2.70
VI–VII	1.17±1.08	1.08	0.054±0.050	1.08
II–I	2.10±1.03	2.04	0.081±0.045	1.80
III–I	5.34±1.21	4.41	0.189±0.054	3.50
III–II	3.24±1.15	2.82	0.108±0.052	2.08

were performed during 1941. Male White Leghorn chicks were used. They were hatched on March 7, operated on at the age of 46 days (April 22), and killed 7 months after operation (between November 24 and 28). There were 7 groups, as follows: *I*, comb removed; *II*, wattles removed; *III*, comb and wattles removed; *IV*, edges of comb and wattles clipped; *V*, posterior half of comb cut off; *VI*, three deep horizontal cuts made into comb; *VII*, control. Each group was started with 70 chicks. Very few chicks were lost from disease, but many were taken by foxes, so that the final number in the different groups was between 44 and 53. Feeding and management were, of course, the same for all groups.

The means of body weight, testicular weight, and testes weight relative to weight

of the body, together with their standard errors, are reproduced in table 2. Table 3 shows the differences in actual and relative mean testes weights between the control and the six operated groups and also between some of the experimental lots.

It can be seen that, in comparison with the controls, the actual and relative testes weight was significantly increased in *Groups II, III* and *V*. An enlargement of doubtful significance occurred in *Group IV*. *Groups I* and *VI* did not differ significantly from the controls.

It is obvious that some of our experimental procedures led to increased testicular weight, and in this respect our results tend to confirm those of earlier investigators. Is one justified, however, in concluding that this proves that removal of the head

TABLE 4. MEANS AND STANDARD ERRORS OF CORRECTED BODY WEIGHT AND OF RELATIVE TESTES WEIGHT BASED ON CORRECTED BODY WEIGHT, TOGETHER WITH THE DIFFERENCES IN MEANS BETWEEN EXPERIMENTAL GROUPS AND CONTROLS

Group	I	II	III	IV	V	VI	VII
Number of birds	48	46	48	45	53	44	52
Corrected body weight, gm.	2139.1±33.98	2201.7±25.86	2225.9±29.16	2161.1±31.99	2200.9±35.98	2137.4±39.18	2110.4±31.16
Difference of corrected body weight from control, gm.	28.7±46.10	91.3±40.49	115.5±42.68	50.7±44.66	90.5±47.60	27.0±50.06	
Testes weight×100 corrected body wt.	0.418±0.0340	0.505±0.0306	0.644±0.0417	0.437±0.0314	0.450±0.0303	0.390±0.0394	0.336±0.0278
Difference of corrected relative testes weight from control	0.082±0.0439	0.168±0.0413	0.308±0.0501	0.101±0.0419	0.114±0.0411	0.054±0.0482	

appendages has a direct causal effect upon weight of the testes? The evidence needs further scrutiny before such an interpretation can be accepted.

The data on relative testes weight in table 2 were calculated on the basis of the actual weights at the time of killing. The same method appears to have been followed by other authors. It is clear, however, that this is not a correct procedure since these body weights in some cases, *e.g.*, the controls, contain the weights of comb and wattles, whereas in other groups they include but one, or only parts, or neither of these appendages. It was decided, therefore, to calculate 'corrected' body weights by subtracting in each case the weight of the appendages, if any, from the gross body weight and to recalculate relative testes weight on the basis of these corrected body weights. The resulting data are given in table 4. In *Groups II, III* and probably also in *Group V* the relative weight of the testes again is significantly higher than in the controls, whereas *Groups I, IV* and *VI* do not show significant differences.

The greatest increase in relative (and absolute) testes weight is shown by the cockerels of *Group III*. This is the one group in which the body weight is significantly higher than in the controls. In the two other groups with a significant increase of relative testes weight the mean body weight is also above that of the controls, although in these instances the differences amount to only about twice the standard error. On the other hand, the three groups, in which the relative testes weight did not differ significantly from the control mean, showed no significant deviations from the controls in mean body weight.

In order to complete the picture of changes in body weight, the mean weights at the time of operation are given in table 5. There was at that time no significant difference between any two of the seven groups.

DISCUSSION

The genesis of testes enlargement after removal of the head appendages has been variously interpreted by different authors. Zawadowsky (2, 3) and Zawadowsky and Slawina (7) suggested that the head appendages produce a substance which inhibits growth of the testes. In addition, Zawadowsky (3) considered the possibility that by removing heat-losing surfaces (the head appendages) a change of body temperature is produced which in turn is reflected in testes enlargement, but he was unable to find corroborative evidence. Bissonnette (8) suggested that removal of the comb meant an increase in the reception of sexually stimulating light. Hoskins and Koch (4) invoked a triangular interrelationship between comb, pituitary and testes. Other

TABLE 5. MEANS AND STANDARD ERRORS OF BODY WEIGHT OF WHITE LEGHORN COCKERELS AT SIX WEEKS

Group	Number of Birds[1]	Mean, Gram
I	47	339.6±6.65
II	45	340.4±5.40
III	46	346.6±6.27
IV	45	335.1±6.78
V	53	347.5±7.24
VI	44	340.7±7.80
VII	51	338.4±5.57

[1] Only the cockerels which came to autopsy are included in this tabulation. A few of the survivors had lost their identification bands; hence, their earlier body weight could not be established.

investigators wisely refrained from giving a causal interpretation of their findings.

We believe that speculations such as those to which reference has just been made are quite unwarranted, and that a much simpler explanation of the observed facts is at hand.

All available experimental evidence, except that reported by Zawadowsky, was obtained by removing the head appendages of young cockerels and by comparing the testes of these animals, after a certain period of growth, with those of the unoperated controls. In our own material a close correspondence exists between body weight and weight of the testes, as is illustrated by the following sequences of increasing mean weights in the seven different groups:

Corrected body weight: VII <VI <I <IV <V <II <III
Actual testes weight: VII <VI <I <IV <V <II <III
Relative testes weight: VII <VI <I <IV <V <II <III

It is of particular interest that the group in which the whole comb was removed (*I*) did not show an increase in body weight,—nor one in testes weight. In earlier experiments, for which the detailed data are not given here, it was also observed, without exception, that increased testicular weight occurred in the experimental groups only when body weight was greater than in the controls.

A scrutiny of the data of other investigators points to the same relationship. Especially illuminating are the observations by Hoskins and Koch (4). Among their five series of experiments two produced appreciable differences in testes weight (table 1). In one instance the controls had the larger testes weight, in the other instance the experimental animals, but for both cases it is true that the group with the larger ultimate testes weight had made the greater gain in body weight between the time of operation and termination of the experiment. Marlow and Payne (5) as well as Nikolaiczuk and Maw (6) found that body weight was increased in the animals from which head appendages had been removed.

All these facts taken together leave no doubt that the removal of part of or all the head appendages may stimulate body growth and that the increase in testes weight occurs secondary to or parallel with this effect. It is true that the gain in final body weight is not very great in any experimental group, but it must be remembered that the correlation between growth in body weight and growth in testicular weight is non-linear and that a relatively small gain in physical maturity may have a conspicuous effect on the size of the gonads. On the other hand, it is possible that the same agencies which are responsible for the attainment of increased body weight act simultaneously on growth of the testes. This latter alternative is suggested by the fact that increased testicular weight, after removal of head appendages, can be obtained in mature cocks as well as in growing cockerels (Zawadowsky).

Observations which are of considerable interest in this connection have been reported by Steggerda (9–11). This author found that ovarian injury or simply bleeding from a wing vein may cause an earlier onset or a stimulation of initial rate of egg production in pullets. He got these results by operating on young female chicks (age 6 weeks) as well as by using pullets which were 4.5 months old. Unpublished observations of our own show similar results. When head appendages were removed from female chicks at the age of 6 weeks a slight increase in mean ovary weight was found in one group at the age of 5 months and these particular pullets also showed a significantly higher body weight than the controls. Again, in other experiments (involving approximately 120 birds) it was observed that removal of the comb or of both comb and wattles from pullets which were just approaching maturity resulted in a somewhat earlier beginning of laying and a slightly higher rate of egg production during the first few weeks after maturity had been reached. There was no demonstrable difference in total egg production. This experiment was repeated with similar results.

All data which have been discussed in this report are consistent. Their understanding does not require the assumption of a s p e c i f i c influence by the comb or wattles on the gonads. The problems clearly belong to the field of traumatology rather than that of endocrinology in the narrow sense, although endocrine relationships of a more general kind may, of course, be involved. We do not know the mechanisms by which the initial trauma leads to an ultimate gain in weight of body and testes. They are likely to be after-effects of the complex changes which are known to be produced by traumatic shock and by the consequent 'alarm reaction' as it has been discussed by Selye (12, 13). It is in good harmony with such an interpretation that, according to Nikolaiczuk and Maw (6), removal of the head appendages is at first followed by retarded physical development and only later results in excess gains of body weight.

A final word needs to be said in regard to growth of one of the head appendages, comb or wattles, if the other one has been removed. Buckner, Insko and Martin (1) reported that after comb removal the wattles exceeded in size those of the controls, and they interpreted this wattle enlargement as "probably compensatory for the removal of the combs and diminished total surface area which apparently is sensitive to the direct radiant energy of the sun." Nikolaiczuk and Maw (6), on the other hand, made the statement that there was no compensatory wattle development after removal of the comb. In our experience comb weight after removal of the wattles or wattle weight after removal of the comb generally did not differ significantly from the weights in the control groups. In at least one case, however, a clearly significant increase in comb weight was found after ablation of the wattles. Yet, there is no reason to invoke compensatory hypertrophy for explanation, since increased comb size

occurred in a group with superior weight of body and testes. Enlargement of the remaining appendages, when it occurs, appears to be merely an expression of the gonad enlargement.

SUMMARY

It is confirmed that removal of one or both head appendages from cockerels may be followed by increased testicular weight. Such a response by the testes occurs only if body weight is greater than in the control animals. There is no evidence whatever for a specific effect of comb or wattles on size of the testes. Instead, the present data as well as those reported by others indicate that the causative agency rests with the trauma produced by removal of the head appendages.

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INFLUENCE OF SOME STEROID HORMONES ON LACTATION IN ADRENALECTOMIZED RATS[1]

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PREVIOUS WORK has shown that the adrenal cortex is necessary for normal lactation (literature cited in reference 1, 2) and recently it has become a matter of interest to identify the hormones of cortical adrenal origin which can support lactation. It has been reported that desoxycorticosterone acetate (1) will not maintain normal lactation in adrenalectomized rats and that qualitative differences exist in certain types of extracts of the adrenal cortex (3, 4, 5). Spoor, Hartman and Brownell (5) have recently given additional evidence that a fraction not defined chemically but designated as cortilactin, can be separated from adrenal tissue, which when combined with a life-maintaining factor permits normal lactation in rats after adrenalectomy. Cortilactin also stimulated crop glands of pigeons and is prepared in part by the methods used to isolate pituitary lactogen.[2]

In this report it will be shown that in adrenalectomized rats essentially normal lactation is maintained by 17-hydroxy-11-dehydro-corticosterone (compound *E*). A further study of the response to desoxycorticosterone, adrenal extracts and the sex steroids is also reported here.

METHODS

Experiments were conducted as previously described (1) Rats approximately 225 gm. in body weight were adrenalectomized within 24 hours after parturition, and all litters were reduced to 6 young each, 3 males and 3 females. Treatments were started immediately after the operation and were given daily for 17 or more days, but not beyond the 22nd day. At about the 17th day the young rats began to eat and it might be expected that a normal growth curve would be easy to maintain thereafter. Actually, however, we have been unable to find any treatment that would consistently maintain a normal growth curve between the 17th and 22nd days and continued therapy during that period did not seem to improve results. For that reason treatment was stopped at the 17th day in some later cases and it is the figure for that day which is strictly comparable in all series (fig. 1).

Identical daily doses could not be obtained for the various substances given in the drinking water, but rations of hormones[3] were prepared daily on the basis of probable consumption and could be made to approximate closely the desired amounts.

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[2] While this paper was in press the existence of an adrenal substance with the crop gland stimulating properties attributed to cortilactin has been questioned (13).

[3] The desoxycorticosterone acetate (Cortate) and testosterone propionate (Oreton) were kindly supplied by Dr. Erwin Schwenk, Schering Corp., Bloomfield, N. J.; and the estradiol-17-propionate by Dr. E. W. Blanchard, Schieffelin and Co., New York City. The extracts and crystalline steroids of the adrenal cortex were prepared in the laboratories of the Mayo Foundation.

RESULTS

Normal lactation in this colony, as judged by the rate of growth of litters from untreated control mothers is indicated in table 1, *series* 1. The strain of animals used has been long inbred in this laboratory and the growth curve, frequently checked, has remained essentially constant during the last 8 years. The animals are relatively small and at no age throughout life reach the size of some strains.

Lactation in untreated adrenalectomized animals is indicated in table 1, *series* 2. Results are almost identical with those previously published (1). Normal growth

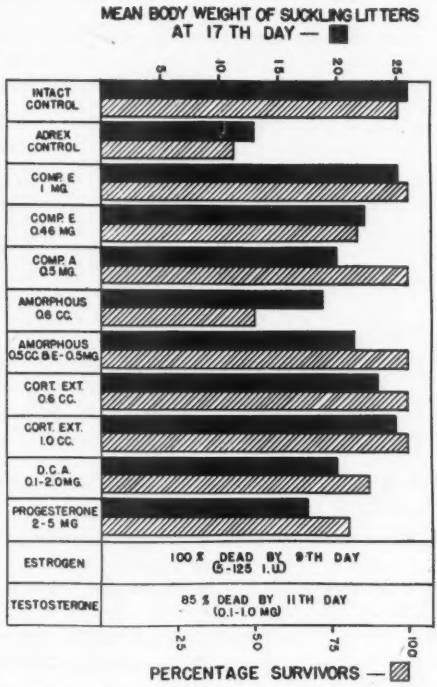

Fig. 1. Body weight at 17th day and survival of litters of adrenalectomized rats receiving various treatments

never occurred. The lactating mothers survived adrenalectomy well and none died before their young were old enough to eat.

Compound A (*dehydrocorticosterone*), dissolved in sesame oil at a concentration of 4 mg. per cc., was given to 4 adrenalectomized mothers in daily doses of 0.5 mg. No deaths occurred in the suckling young. Normal growth was not achieved although the treatment was clearly beneficial, (table 1, *series* 5).

Compound E, dissolved in acetone and sesame oil, in a concentration of 4 mg. per cc., was given in 0.5 mg. daily doses to 3 lactating mothers. In 3 other cases the material was given in similar dosage dissolved in the drinking water. No differences were noted for the two routes of administration and the results were tabulated together, (table 1, *series* 4). One litter died, but the remaining ones were all obviously benefited, although normal lactation was not mainatined.

When, however, 1 mg. of compound *E* was given in the drinking water a completely normal lactation was maintained for the first 17 days, (table 1, *series* 3). There was, as discussed above, a slight but consistent drop below normal between the 17th and 22nd day, after which the growth curve reassumed a normal slope.

The amorphous fraction was given to 4 lactating mothers in 0.6 cc. daily doses in drinking water. Two of the litters died and the growth curves of the surviving litters, although fair, should therefore probably be discounted. The material was presumably of little if any benefit at that dose level, (table 1, *series* 6).

When 0.5 cc. of the amorphous fraction was combined with 0.5 mg. of compound

TABLE 1. RESPONSE OF ADRENALECTOMIZED MOTHERS AND THEIR SUCKLING YOUNG TO VARIOUS TREATMENTS

Treatment	Number of Litters Used	Daily Dose, mg. or cc.	Average Body Weight of Surviving Suckling Young at Day								Surviving Young, %	Weight Change in Mothers While Treated, %
			1	5	10	15	17	20	22	24		
1. Control, untreated	18		5.5	9.5	16.5	23.0	26.0	32.0	36.0	41.0	97	+ 4.2[1]
2. Adrenalectomized, untreated	16		6.0	9.3	12.2	12.7	13.0	14.1	15.6	18.2	43	− 5.0[2]
3. Compound E	5	1.02	6.1	10.6	16.9	23.0	25.2	28.6	32.2	38.4	100	− 0.8
4. Compound E	6	0.46	5.9	9.3	13.9	19.0	22.2	25.0	29.3	35.1	83	+ 7.4
5. Compound A	4	0.5	6.2	9.1	14.5	18.7	20.0	22.1	25.2	29.0	100	+11.4
6. Amorphous fraction	4	0.6	5.9	8.9	12.6	15.4	18.8	21.3	25.6	29.6	50	0.0
7. Amorphous fraction + compound E	4	0.45, E 0.5 Amor.	6.2	9.6	14.6	19.6	21.4	23.9	26.4	31.6	100	+ 2.2
8. Cortical extract	7	0.6	6.2	10.9	16.6	21.6	23.3	26.9	31.9	36.6	100	+ 0.8
9. Cortical extract	4	1.0	6.0	10.1	16.1	22.2	24.6	28.7	35.2	40.5	100	0.0
10. Desoxycorticosterone acetate	10	0.1–2.0	5.8	9.4	14.4	18.6	20.4	24.0	27.7	31.8	87	+ 8.4
11. Progesterone	8	2 or 5	6.0	8.3	12.4	18.3	19.9	20.8	23.9	27.4	87	− 2.7
12. Estradiol	9	5–125[3]	5.8	7.2							00	− 5.2
13. Testosterone propionate	7	0.1–1.0	5.7	8.0	8.8						14	+ 3.6

[1] During first 20 days of lactation. [2] While lactating. [3] I.U.

E as a daily dose in drinking water, the results were no better than those anticipated with compound *E* alone, (table 1, *series* 7 and 4.)

Further supplies of the amorphous fraction are not presently available for tests at higher dose levels.

Whole extract of the adrenal cortex (75 gm. of the gland, per cc.) was tried at two dosage levels. It was much more effective than the amorphous fraction for the maintenance of lactation at a similar dose level (0.6 cc.). One cc. doses supported a growth curve that approximated normal except for the customary slight drop around the 20th day, (table 1, *series* 8 and 9).

Desoxycorticosterone acetate was given to 10 adrenalectomized mothers. One-tenth mg. daily was given in 4 animals, 1.0 mg. in 4 and 2 mg. in 2 animals. No differences could be noted between the effects of the various dosages, and so all of these are figured together, table 1, *series* 10). The results were so variable for each dosage level, however, that a mean has little significance. Of the mothers receiving 1.0 mg. daily, parts of the litters of two starved and the other two approached but did not reach normal growth. Of the two receiving 2 mg. daily, one litter again almost starved and the other, while not normal, grew fairly well. Results with other crystalline materials were always more consistent.

Estrogen (*estradiol-17-propionate*) exerted its characteristic lactation-inhibiting effect in smaller doses in adrenalectomized than in intact animals. One mother received 125 I.U. per day, 4 received 10 I.U. and 4 were given 5 I.U. All litters were dead by the 9th day, (table 1, *series* 12). Comparable studies on intact animals of this colony were made by Edelmann and Gaunt (6).

Testosterone propionate likewise inhibited lactation in small doses, although it, unlike estrogen, is not toxic to adrenalectomized rats and in only 1 of 7 treated mothers was there a loss of weight during the period of treatment. Two mothers received 1 mg. daily, and their litters survived 8 days; the litters from two receiving 0.25 mg. were all dead by the 11th day; 3 mothers received 0.1 mg. and one litter only survived, (table 1, *series 13*.) In intact animals doses as high as 3 mg. per day do not bring about death of all litters (6).

Progesterone was given to 6 adrenalectomized mothers in doses of 2 mg. daily. One litter died and the others apparently received some benefit from the therapy. Dosages of 5 mg. gave similar results. It is significant that even in these sensitive animals progesterone clearly was not an inhibitor of lactation (table 1, *series 11*). Previous studies with this compound in adrenalectomized lactating rats under different conditions have been made by Tobin (7).

DISCUSSION

The results indicate that lactation in adrenalectomized mothers was benefited by practically all agents known to relieve adrenal insufficiency although there is question concerning the small series treated with the amorphous fraction. It was easy to increase milk secretion considerably above the deficient levels of untreated adrenalectomized animals, but exceedingly difficult to maintain actually a fully normal lactation. Especial significance is therefore assigned the substances (compound *E* and cortical extract) which can do so.

Desoxycoticosterone acetate has given the most inconsistent and ill-understood results. Previously one of us reported (1) that this material maintained life in only one of 14 suckling litters, and that this one 'anomalously' grew normally. Our present series indicates that a higher percentage of young receive benefit when the mothers receive desoxycorticosterone acetate, but, as pointed out in a previous section, the beneficial effects are not invariable and clearly are not proportional to the dosage. In a total of 24 litters whose mothers received desoxycorticosterone acetate in this and the previous experiments, no litter reached the mean control body weight at the 17th day, and only one at the 24th day. Some, however, were in the lower ranges of normal. We consider it probable therefore that desoxycorticosterone acetate may assist some animals in supporting lactation only insofar as lactation is improved by the maintenance of general well-being and health in the mother. The administration of sodium chloride gives somewhat similar results (8, 9).

The inadequacy of desoxycorticosterone acetate is also indicated by the work of Nelson (10) who found that it, unlike cortical extract, did not offer the supplement necessary to permit lactogenic hormone to initiate lactation in the hypophysectomized guinea pig.

If one interprets these results in terms of the best-known action of desoxycorticosterone acetate (on electrolyte metabolism) and of compound *E* and its relatives (on carbohydrate metabolism), it could be concluded that the restoration of a more or less normal electrolyte metabolism is helpful in the maintenance of lactation but is not self-sufficient. Normal lactation is probably the result of the combined activities of more than one of the hormones of the adrenal cortex with a critical dependence for maximal mammary secretion upon those substances known principally for their effects upon carbohydrate metabolism. Only with compound *E* and with cortical extract have we consistently maintained lactation around the normal mean value.

In support of this general idea, work now in progress with hypophysectomized guinea pigs indicates that compound *E* is a highly effective supplement to pituitary lactogen for the initiation of lactation while, as previously mentioned, desoxycorticosterone acetate is probably ineffective.

While such interpretations will fit the data offered here, they are difficult to harmonize with the results of Spoor et al. (5) who find that the cortilactin, which has no effect on gluconeogenesis, will, when supplemented by a life-maintaining substance, maintain normal lactation. This again suggests that the support of normal lactation is the combined function of different cortical substances but it does not imply the necessity of a 'carbohydrate factor.'[8]

Previous results indicated the possibility that desoxycorticosterone acetate was a lactation inhibitor in the dosages used here, but the present experiments would seem to eliminate that possibility. We have also been unable to inhibit lactation in intact animals with 2 mg. of desoxycorticosterone acetate daily (2 litters). In the guinea pig, which is more sensitive than the rat to the lactation-inhibiting effects of estrogenic steroids, desoxycorticosterone acetate does have an inhibiting action (10).

The question of whether progesterone could inhibit lactation has been one of some theoretical interest. Folley and Kon (11) were unable to inhibit it in intact rats with 1 mg. of progesterone. In adrenalectomized rats, which seem to be more sensitive than intact ones to the lactation-inhibiting effects of estrogen and androgen, progesterone even in large doses still did not inhibit lactation. On theoretical grounds it might be considered unlikely that progesterone would act to nullify the effect of lactogenic hormone, since according to recent findings (12, and others) it is the lactogenic hormone which itself maintains the secretion of progesterone. The complexities of endocrine relationships are such, however, that one could not safely depend upon the validity of such reasoning without experimental proof.

SUMMARY AND CONCLUSIONS

The effects of various steroids of the adrenal cortex and the gonads on the deficient lactation of adrenalectomized rats were studied. There were indications that all substances known to relieve adrenal insufficiency were somewhat helpful, presumably because of their maintenance of a generally improved health (electrolyte metabolism?).

Desoxycorticosterone acetate gave variable results; it was helpful in some but not in all animals, and in no case maintained lactation equal to the average control figure.

Normal lactation (for the first 17 days) was obtained only with compound *E* and the whole extract of the adrenal cortex, a fact which suggests that full mammary secretion is dependent on those adrenal factors which affect primarily carbohydrate metabolism. Such a conclusion is only tentative since it is not consistent with all other work on the subject (5).

Estrogen and androgen inhibited lactation even in small doses. Progesterone did not inhibit lactation.

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ESTROGENS, BLOOD SUGARS AND LIVER GLYCOGEN IN NORMAL AND HYPOPHYSECTOMIZED GUINEA PIGS[1]

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THE METABOLIC effects of estrogen therapy in the organism remain relatively confused, especially with regard to carbohydrate metabolism. This paper deals specifically with the relations of the pituitary and of estrogen therapy to blood sugar and liver glycogen in the guinea pig. Over a period of several years one of us (R. T. H.), in the course of treating hypophysectomized animals with estrogens, has accumulated a great number of confounding data. These have been of specific importance for the survival of hypophysectomized animals with subsequent estrogen treatment. These experiences led to the performing of detailed experiments in an effort to clarify this confusion.

EXPERIMENTAL PROCEDURE

Care of animals. All animals were fed Purina Complete Rabbit Chow, with a supplement of fresh lettuce and carrots. Any apparently unwell animal was destroyed. Injections of estrogens (in oil) were made subcutaneously, at the intervals and dosages indicated in table 1. The estrogens used were estrone[2] and estradiol benzoate.[2] All hypophysectomy operations were done under sodium amytal anesthesia, and by the parapharyngeal route (1). The completeness of pituitary ablation was checked at autopsy.

Chemical analyses. All blood samples were removed by cardiac puncture and without anesthesia. This method has proven adequate and reliable, and seemed better than to introduce the factor of anesthesia which is known to influence the sugar content of the blood and the glycogen content of the liver (2). Sugar determinations were made according to Folin's method (3). The animals were killed by decapitation, and liver samples (middle lobe) were removed at once. Pflüger's modified technique (4, 5) was used in glycogen determinations. Liver samples from some guinea pigs were removed and sectioned and stained for glycogen. Such an examination, however, is of only relative value, and data obtained therefrom could offer no increased significance to findings made by chemical analysis for glycogen. The correlation of histologic sections with chemical findings was as good as the methods would allow.

DATA AND DISCUSSION

A sample of the data obtained in these experiments is given in table 1, and in general relate to 3 groups of guinea pigs. One group was hypophysectomized, with subsequent estrogen treatment and fasting; a second group consisted of hypophysecto-

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[1] This investigation has been made possible through a grant from the American Philosophical Society and from the Graduate School of Indiana University.

[2] Estrone and sodium amytal were supplied by Eli Lilly & Co., Indianapolis, Ind. Estradiol benzoate (Progynon-B) was furnished by Schering Corp., Bloomfield, N. J., through the courtesy of Dr. Erwin Schwenk.

mized guinea pigs subjected to fasting only. The third group was unoperated and not fasted, but was injected with an estrogen and was used to supply liver samples for glycogen determinations only. The sex and specific treatment of each animal are given in the table.

During the first few days following pituitary removal it is often necessary to treat the animals with glucose injections to maintain life. Animals found in coma during these few days always have an extremely low blood sugar (35–60 mg. %), and when given glucose the recovery (although it may be temporary) is remarkable. Carrots have a sugar content of about 7 per cent of their fresh weight, and have been found to be just as efficient in postoperative treatment of hypoglycemia as glucose injections. Four or 5 days subsequent to hypophysectomy, the guinea pigs usually re-establish a blood sugar level within the limits of those of normal unoperated animals. Following this they will live for several months with no treatment other than an adequate diet. Whether the animal is male or female does not alter the picture. The blood

TABLE 1. REPRESENTATIVE DATA SHOWING TREATMENT, RESULTANT BLOOD SUGAR CHANGES AND SOME TERMINAL VALUES FOR LIVER GLYCOGEN[1]

Animal Number and Type	Hours Fasted											Liver Glycogen, %
	6	30–38	54	72	104	128	152	176	200	224	248	
	Blood sugar in mg. %											
98 ♀, hypophysectomized	94 *E*, 5	34[2]										0.83
101 ♀, hypophysectomized	119 *E*, 5	70 *E*, 5	95 *E*, 5	100[2]								0.6
115 ♂, normal	102 *P*, 20	89 *P*, 20	117 *P*, 20	121[2]								0.57
132 ♂, normal	107 *P*, 10	99 *P*, 10	106 *P*, 10		85 *P*, 10	94 *P*, 10	81 *P*, 10	84 *P*, 10	83 *P*, 10	93 *P*, 10	89[3] *P*, 10	
134 ♀, hypophysectomized	102 *P*, 10	115 *P*, 10	97 *P*, 10		130 *P*, 10	92 *P*, 10	80 *P*, 10	79 *P*, 10	50 *P*, 10	41 *P*, 10	40[3] *P*, 10	

[1] Data were obtained from more than 140 hypophysectomized guinea pigs, and a large number of controls. Injections of either estrone or estradiol benzoate were made every 24 hours in most of the experiments. The number following the letter (i.e. *E*, 5 signifies the thousands of I.U. injected; *E*, estrone; *P*, estradiol benzoate.
[2] Killed at point of death.
[3] Killed.

sugars of all hypophysectomized guinea pigs used in these experiments had become stabilized before any treatment was started.

In these experiments estradiol benzoate and estrone were injected (oil solutions) subcutaneously, and blood samples were taken at regular intervals for sugar determinations. The blood sugar level of normal guinea pigs varies rather widely, from a low of around 90 to a high of about 130 mg. per cent. This range is somewhat lower than Smith et al. (6) found for monkeys. This normal variation must be kept in mind. In no case did the blood sugar of an animal with an intact pituitary suffer from the treatment by estrogens during a fasting period of 72 hours (see table 1).

On the other hand the hypophysectomized guinea pigs undergoing identical treatment for 72 hours fall into two types. One group entered the experiments with a normal blood sugar level, and reacted in a manner similar to that of the intact animals. The only point of deviation in the response of these guinea pigs from that of the intact ones was that most of them had exhibited a state of definite hypoglycemia at one time during the experiment, followed by recovery to an essentially normal level.

The second group of operated guinea pigs likewise entered the experiments with normal blood sugar levels. These, however, soon exhibited a hypoglycemic condition from which they did not recover. This condition either led to death or to a state of

coma. When an animal was found in coma, a last blood sample was taken and then the animal was killed to get a liver sample for glycogen tests.

When the fasting period was increased to a total of 248 hours, a very similar picture was found. The animal with the pituitary intact at the end of the experiment had a blood sugar content of 89 mg. per cent. Two estrogen-treated hypophysectomized guinea pigs were found dead after having shown blood sugars of 38 and 52 mg. per cent, respectively. A third died after a blood sugar of 102 mg. per cent at the end of 104 hours. The cause of death in this animal was the result of an injury while taking a blood sample. A fourth animal lived the entire 248 hours, but at the end of the experiment had a blood sugar value of 40 mg. per cent, as compared with 89 mg. per cent in the intact animal at the end of the same interval.

When guinea pigs, both normal and hypophysectomized, were fasted for 72 hours (no estrogen treatment) not a single fatal case of hypoglycemia resulted. In fact all animals reacted very similarly regardless of the presence or absence of the pituitary.

Liver-glycogen determinations made on samples taken from most of the foregoing guinea pigs were all of very low value, with an occasional unexplainable exception. We do not believe that, beyond the low values found, any significance exists in the data or in its variation from one animal to another.

However, all liver-glycogen determinations made on samples from estrogen-treated normal animals gave much higher values, about four times as great as those in untreated normal or hypophysectomized guinea pigs fasted for 72 hours. This difference is at least partly explainable on the basis of the longer fasting period (72 hours as compared with 6 to 24 hours). The liver glycogen values obtained from intact, untreated and unfasted animals were very similar to those of the intact estrogen-treated group.

No sex differences with regard to blood sugar levels were found following the treatment of operated animals with estrogens. Both sexes appeared to follow the same reaction pattern.

Likewise, all glycogen determinations made on liver samples showed no difference between the sexes. Thus, even though the problem still remains one of a female sex hormone treatment of an hypophysectomized guinea pig, the sex of the treated animal does not enter into the complex.

There is considerable evidence pointing to a diabetogenic activity of the anterior lobe of the pituitary (7, 8, 9). Pituitary removal would be expected to disturb the sugar and glycogen balances of the body, and these experiments add additional evidence relative to blood sugars. Further, it appears that the guinea pig, after hypophysectomy, can re-establish the control of blood sugar in a fairly normal fashion. The subsequent introduction of either one of the two estrogens used in these experiments has a tendency to again unbalance this control. Some animals obviously can compensate for this factor, while others cannot, and thus estrogen treatment may become a lethal factor through production of hypoglycemia. The only liver-glycogen disturbance noted in these experiments seemed to be correlated more with the length of the fasting period than with hypophysectomy and estrogen therapy.

It is doubtful if such sugar changes as were observed can be assumed to follow an intermediary rôle played by the pancreas, or if such changes were the result of a direct relationship between the pituitary and estrogen. Insofar as the day to day changes of blood sugar in estrogen-treated intact animals were quite within the limits of normal variation, it would seem that in those animals no significance can be attached to the treatment beyond the effect of the fasting period. Fasting alone appeared to cause a general lowering of blood sugars in the operated animals (20–30 mg. % lower), but

the picture is different from that obtained when similar animals were treated with estrogens.

In experiments on hypophysectomized monkeys (6, 10) it was found that the fasting blood sugar was markedly different from that of normal monkeys. Results with the use of insulin in these animals seem to suggest that the absence of the pituitary and its diabetogenic function may be directly responsible for hypoglycemic shock or coma developed in normal monkeys.

Recently Evans and co-workers (11) have found an increased insulin output of the pancreas in normal rats treated with estrogen, but not in hypophysectomized estrogen-treated rats. If the same is true in guinea pigs, then it would seem to follow that the blood sugar disturbances presented here might be from a direct effect of estrogen metabolism in hypophysectomized guinea pigs. It is particularly worthy of note that these authors refer to the general intolerance of hypophysectomized rats for estrogen treatment. This observation is identical with ours, as stated earlier in this paper.

SUMMARY

Estrone and estradiol benzoate, when injected subcutaneously in oil solution into hypophysectomized guinea pigs, cause a disturbance in the blood sugar picture. Some individuals are able to recover from the hypoglycemia produced, while in others it proves fatal. Liver glycogen does not seem to be affected. Our evidence lends further support to the already existing data dealing with an anterior lobe factor of blood sugar control. Although the female sex hormones were used in these experiments, the sex of the experimental animal does not affect the final analysis.

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EFFECTS OF CRYSTALLINE ESTRIN IMPLANTS ON THE TIBIA OF YOUNG HYPOPHYSECTOMIZED FEMALE RATS

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A REPORT HAS RECENTLY been made by this laboratory describing the effect of implants of crystalline estrin on the tibia and costo-chondral junction of young normal female rats (1). After three weeks of estrinization, histological examination of the tibia revealed thick trabeculae that filled the upper fifth of the diaphyseal marrow cavity.

Due to our interest in the endocrine factors influencing skeletal growth, which had previously been centered on the rôle of the hypophyseal growth hormone in young normal and hypophysectomized rats (2, 3, 4), we were much interested in the rôle of the hypophysis in the reaction described above. Therefore, a parallel experiment was performed on hypophysectomized rats.

MATERIALS AND METHODS

Twenty female rats were hypophysectomized at the ages of 54 to 58 days, 10 to be treated with estrin and 10 to serve as controls. Treatment, beginning on the day of operation, consisted of subcutaneous implantation of pellets of α-estradiol dipropionate[1] (10 mg.), 1 pellet weekly for 3 weeks. All hypophysectomized animals received 1 cc. of 20 per cent glucose daily intraperitoneally. Ten normal animals were maintained as controls. The experiment was planned to run for 21 days (in order to parallel exactly the normal group treated for three weeks as described in our earlier report 1), but due to the poor condition of the group receiving estrin, the experiment was terminated 18 days postoperatively.

Weight changes from the day of operation to the day of autopsy were recorded. Histological preparations of the tibia were made in a manner similar to that described previously (1).

RESULTS

No histological differences were noted between the tibia of the hypophysectomized rats treated with estrin and those of the untreated.

Figures 1 and 2 illustrate the sub-epiphyseal zone in the proximal portion of the tibia of treated and control hypophysectomized rats, while figures 3 and 4 show the histological appearance of the corresponding region in implanted and untreated nor-

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[1] The α-estradiol dipropionate was supplied by Ciba Pharmaceutical Products, Inc. Summit, N. J.

mal rats. The animals were almost completely comparable in age and estrin treatment. The normal series were described in the earlier report (1). Descriptions of the histology of the tibia of young hypophysectomized rats of the age used here have been reported by this laboratory before (2, 3, 4). It will be noticed that the typical changes observed in the normal rat under the influence of estrin, namely, an increase in the number and size of trabeculae making up the primary spongiosa, is not seen here. It will be noted here, on the contrary, that the typical resorption of the primary spongiosa and retention of secondary spongiosa that follows hypophysectomy is apparent in both the treated and control hypophysectomized rats.

The data of the weight changes during the experimental period (table 1) indicate a toxic effect of the high levels of estrin administered. It is true that untreated rats as well as treated ones lost weight, but the weight loss of the estrinized rats was excessive. An average of 35 gm. was lost by experimental rats as compared with 12.5 gm. in the controls, the latter being a weight loss that is usual after hypophysectomy at this age. As in the experiment with estrinization of normal rats, reduction in food intake was observed.

TABLE XI. EFFECT OF ESTRINIZATION[1] ON HYPOPHYSECTOMIZED RATS[2]

Treatment	Body Weight					
	Operation		Autopsy		Gain	
	Average, gm.	Range, gm.	Average, gm.	Range, gm.	Average, gm.	Range, gm.
Hypophysectomized and estrin	150	140–170	114	(95–140)	−36	(25–45)
Hypophysectomized controls	142	118–167	129	(110–154)	−13	(4–27)
Normal controls	172	131–210	205	(152–235)	+34	(21–60)

[1] Alpha-estradiol dipropionate one 10 mg. pellet weekly, 3 weeks, implanted subcutaneously.
[2] Rats hypophysectomized at 54 to 58 days of age; implants and injections begun on day of operation. Autopsy 18 days postoperatively (normal controls 78–84 days of age).

DISCUSSION

It can be seen at a glance that, in the series of animals examined, nothing resembling the effects of estrin on the skeletal histology of normal rats could be demonstrated in the absence of the hypophysis. Since only one age, one dose level and one period of administration was used, no general deductions can be made. It is possible that estrin may bear a different relation to the pituitary in regard to bone structure in other species, just as the nature of the skeletal response to estrin differs markedly in different species. Gardner and Pfeiffer (5) have found that simultaneous administration of androgens and estrogens will prevent the typical estrogen effects on the skeleton of mice, while Pfeiffer, Kirschbaum, and Gardner (6) have shown that similar treatment will not prevent the estrogen effects on the skeleton of the English sparrow.

The evidence reported here points to the hypophysis as part of the mechanism involved in the changes induced in the skeleton of normal rats subjected to high levels of estrin. Further work, namely, the investigation of the rôle of the parathyroid gland in the changes induced by estrin on the skeleton, is necessary before an attempt to explain the effects of estrin can be made. Riddle and Dotti (7) have reported that the serum calcium level of mammals can be raised by sufficiently high doses of estrin but that no rise in serum calcium could be induced in parathyroidectomized rats. It

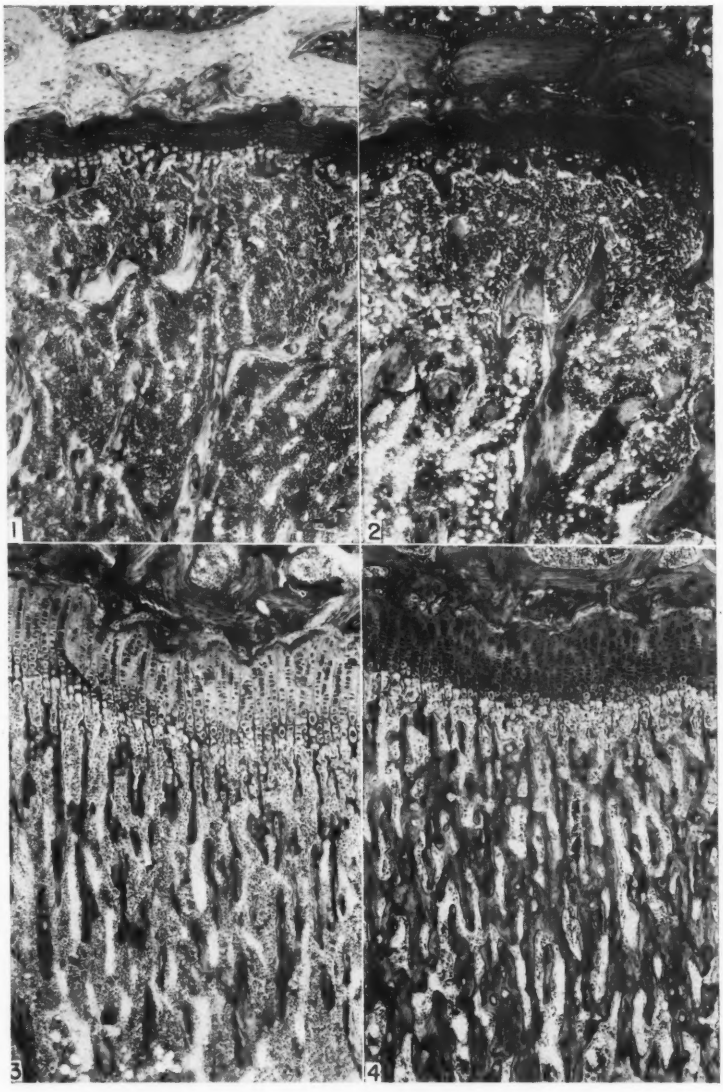

Fig. 1–4. PROXIMAL EPIPHYSEAL CARTILAGE OF RAT TIBIA. Central sagittal section. Hematoxylin and eosin. 10 micra × 100.

Fig. 1. Hypophysectomized at 54th day, 72 days at autopsy. Fig. 2. Hypophysectomized on 54th day, three weekly implants from 54th day, each, 10 mg. of estradiol dipropionate subcutaneously; 72 days of age at autopsy.

Fig. 3. Normal, 76 days of age at autopsy. Fig. 4. Normal, three weekly implants from 54th day, each, 10 mg. of estradiol dipropionate subcutaneously; 75 days of age at autopsy. Figures 3 and 4 reprinted from earlier report (1) in which they were figure 1 and figure 3, respectively.

may be that the mechanism suggested by Zwarenstein (8), that the hypophysis influences calcium metabolism and perhaps skeletal histology through the parathyroid gland, is involved.

SUMMARY

A parallel study of previously reported crystalline estrin implants on the bone histology of normal young female rats (1) was repeated in hypophysectomized rats of the same sex and age. The typical picture of hyper-ossification of the proximal subepiphyseal zone of the tibia was not produced in the absence of the hypophysis.

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NON-EFFECT OF HYSTERECTOMY UPON THE MAMMARY GLAND OF THE MONKEY

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NUMEROUS CLAIMS have appeared in the literature, from time to time, for an endocrine function of the mammalian uterus. Although a functional relationship between the breast and uterus has long been recognized, based on the well known clinical observation that suckling stimulates rhythmical contractions and involution of the puerperal uterus, less convincing has been the evidence of a converse effect of the uterus upon the mammary gland. Foges (1) observed that hysterectomy in young rabbits had no effect on mammary development. Hammond and Marshall (2) have likewise found the uterus unessential for development of the glands, for they observed that mammary hypertrophy could take place in rabbits from which the uterus had been removed while the animals were still immature. In this species the uterus has been found unnecessary, too, for the mammary response to hypophyseal extracts (3). Human cases have also been observed in which good mammary development occurred despite congenital absence of the uterus (4). In the bitch, Turner and Gomez (5) have described the characteristic lobule-alveolar development of pseudopregnancy in the absence of the uterus.

Yet recent experimental studies have furnished evidence suggesting the importance of the uterus in mammary physiology, for the former has been shown to play a prominent rôle in the metabolism of the ovarian hormones. Pincus and Zahl (6) Smith and Smith (7), and Grumbrecht and Loeser (8) have stressed the importance of the uterus in the interconversions and utilization of estrogens by the body. Hamblen, Ashley, and Baptist (9) have pointed out the significance of this organ for the metabolism of progesterone and its excretion as pregnanediol, although more recent observations by Jones and Te Linde (10) have brought into question the findings of the latter authors. Bradbury (11) has also observed the uterus of the mouse to be of importance in the gonad-breast relationship, for prolonged luteinizing treatment was found to be without effect on the mammary glands of hysterectomized mice, in contrast to intact animals, unless some interlobular tissue was already present at the time of operation.

In some species removal of the uterus has been observed to cause a prolongation of the life of the current corpus luteum. Loeb (12, 13) has demonstrated this in the guinea pig and has found a concomitant growth of the mammary glands, with formation of new alveoli and lobules. This proliferation he attributed directly to corpus luteum activity. Hysterectomy in the pseudopregnant rabbit also results in prolonged preservation of the corpus luteum, but no further mammary development occurs beyond that normally attained in pseudopregnancy (14, 15), although Loeb and Smith (16) claimed a delayed involution of the glands which could be correlated with the retrogression of the corpus luteum. Hartman (17) was unable to confirm in the opossum the

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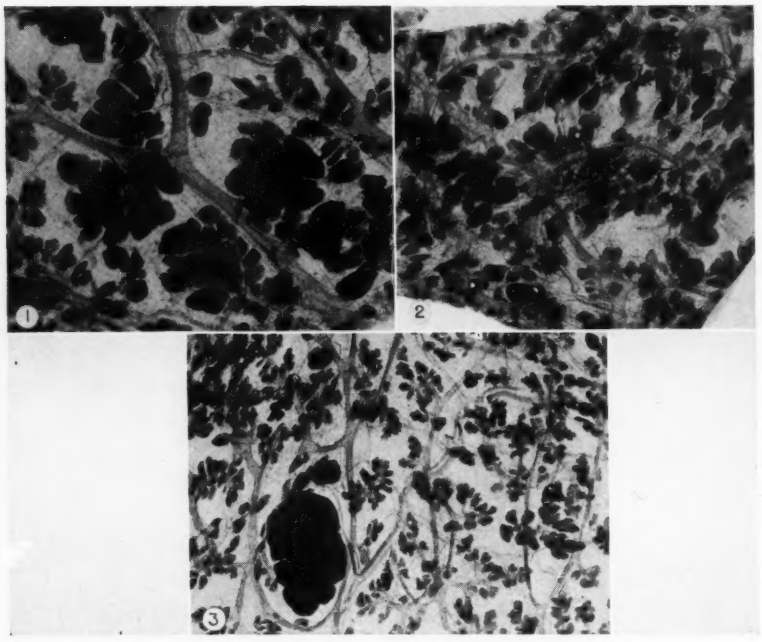

(Whole Mounts; $\times 6\frac{2}{3}$)

Fig. 1. MAMMARY GLAND BIOPSY SPECIMEN 5 DAYS AFTER HYSTERECTOMY, *day* 22 of an ovulatory cycle. Lobules are well developed and show maximal corpus luteum effect.

Fig. 2. SAME ANIMAL 30 DAYS AFTER HYSTERECTOMY. Mammary lobules have undergone retrogression, indicating absence of functional corpus luteum.

Fig. 3. SAME ANIMAL 45 DAYS AFTER CASTRATION. Note further atrophy of lobules and presence of typical 'castration nodule.'

findings of Loeb, for no significant differences could be demonstrated between the hysterectomized animals and the normal controls in the diestrous involution of the mammary glands. In the rat also, hysterectomy is probably without effect, for no luteal persistence or changes in the estrous cycle result (18, 19).

No studies are recorded of the effects of hysterectomy on the mammary glands of the rhesus monkey. We therefore studied with interest the glands of *monkey* 620 by serial biopsies for one month after operation. Abdominal hysterectomy was performed by Dr. Carl G. Hartman on Dec. 11, 1939, which was *day* 22 of an ovulatory menstrual cycle, and an 11-day old fertilized egg was recovered. Mammary gland biopsy was performed at this time and was repeated 5 days later (Dec. 16; fig. 1). Definite enlargement of the mammary lobules was noted, at the time of the expected maximal corpus luteum effect (20). Biopsies were repeated on alternate breasts on Dec. 22, Jan. 3, and Jan. 10 (fig. 2), 11, 23, and 30 days, respectively, after operation. In the whole mounts of these specimens, not only was no further development of the gland to be seen, but there was unmistakable retrogression and shrinkage of the lobules, corresponding to the involution of the corpus luteum during the subsequent anovulatory ovarian cycle.

These observations are in agreement with the findings of Burford and Diddle (21)

who detected no specific changes in ovarian function, as manifested by vaginal lavage, sex skin, and examination of the ovaries at laparotomy in 5 immature and mature macaques which they followed for 121 to 217 days after total hysterectomy. A protocol in Hartman's well known monograph on reproduction in the rhesus monkey (22) describes in detail the course of *monkey 21* for a year following total hysterectomy. In the absence of the uterus this animal continued to run cycles, as evidenced by the vaginal desquamation. Autopsy at the end of this period showed the ovaries to be in an 'excellent state of preservation.' Thus it seems reasonable to conclude that hysterectomy in the rhesus monkey is not followed by the persistence of a functional corpus luteum and is without effect in promoting growth, or even maintaining, the mammary glands.

Subsequent observations of the animal described in this paper have likewise shown the mammary glands to be unaffected by hysterectomy On April 2, 1940, laparotomy and surgical castration were performed and biopsy was made of the mammary gland. Biopsy was repeated on May 17, 45 days later. No treatment was administered during the interim. As was previously reported in the case of untreated castrates (23), localized hyperplastic nodules had appeared in the gland, in addition to the more diffuse atrophy of castration (fig. 3). These changes were apparently not affected by the absence of the uterus.

CONCLUSION

Hysterectomy in an adult female rhesus monkey failed to delay involution of the corpus luteum and had no effect on the mammary glands, which were observed over a period of six months.

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OBSERVATIONS ON THE TRANSPORT OF TUBAL OVA[1]

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IT IS NOT THE PURPOSE of this paper to review the extensive literature on ovum transport because Westman (1), Parker (2), and Hartman (3) have excellent summaries of earlier theories and investigations. In this report we wish to present some direct observations on the passage of ova through the fallopian tubes of albino mice. These observations show that peristaltic movements rather than cilia are responsible for the transportation of ova through the isthmus and intramural region

TECHNIQUE

Mating was determined by the vaginal plug method. At autopsy, the mesosalpinx was cut in several places so that the tightly-coiled oviduct could be somewhat straightened. The tracts were kept in warm Locke's solution (without glucose) in deep-well slides. Each ovary was checked for eruption cones of ovulation or for corpora lutea, and the position of ova within the oviduct was determined by means of transmitted light. Eggs within the ampulla were easily seen, but when they were in either the isthmus or the intramural region where the musculature is greater, the following technique was necessary. All but a short section of the uterus was cut away, and the tube with its attached ovary was placed on a plain, dry microscope slide. The edge of the cover glass was placed against the ovary for anchorage while the tube was very gently straightened, special care being taken not to injure the muscle fibers. After the cover glass was lowered onto the tube, warm oxygenated Locke's solution was run under the cover and changed from time to time by means of a capillary wick. This simple procedure kept the tube in approximately one focal plane so that the ova were more easily observed and photographed. A slight pressure on the cover glass compressed the tubal walls, thus showing the eggs to better advantage. Care was taken to avoid forcing the eggs from one tubal loop into another or from the tubal papilla into the uterus.

The photographs of these tracts are paper negatives with the exception of figure 9 which is a positive made from an Eastman Wratten M negative.

Anatomical Considerations

As is well known, the coiled oviduct of the mouse lies close to the anterior end of the uterus and is somewhat obscured by masses of fat. The accompanying diagram (fig. 1) shows a typical tube more or less straightened after the mesosalpinx has been severed.

The first part of the tube, the infundibulum, is inserted well within the periovarial sac. Fimbriae, low folds of tissue, form a rosette around the tubal ostium.

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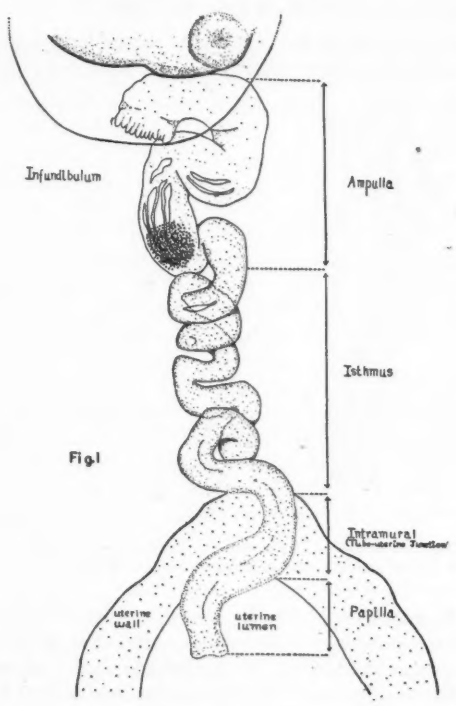
[1] These observations, which cover a period of years, were aided in part by grants from the National Research Council and the Penrose Fund of the American Philosophical Society, administered through the courtesy of the late C. F. Randolph, Treasurer of Alfred University.

The second portion, or ampulla, consists of the first two loops. The second loop is capable of great distention, and serves as a reservoir for the eggs and the fluid from the periovarial sac until some hours after fertilization. Longitudinal folds covered with cilia are clearly seen within the ampulla. The activity of these cilia is particularly persistent and continues even in fluids at room temperature, a fact previously noted by Snyder and Corner (4) and Novak and Everett (5).



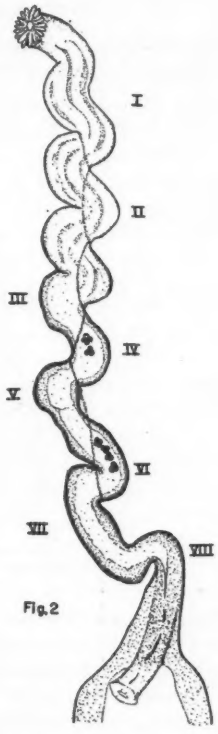


Fig. 1. REGIONS OF A MOUSE OVIDUCT WITH EGGS AND CUMULUS CELLS IN THE SECOND LOOP OF THE AMPULLA. The mesosalpinx has been cut to allow some straightening of the coiled tube. The number and position of loops vary somewhat in different mice. (See fig. 2.)

Fig. 2. MOUSE OVIDUCT SHOWING 4-CELLED EGGS IN TWO DIFFERENT REGIONS. Tubal peristalsis was confined to *Sections IV* and *VI*. Later, *Sections V* and *VII* exhibited peristalsis after the eggs had been propelled into these segments. The dotted line extending along the oviduct shows the position of the severed mesosalpinx. Zonas are still present but do not show in projections.

The third region of the tube, the isthmus, is much narrower than the ampulla and is well supplied with both circular and longitudinal muscles. By pressing the cover glass, the observer may see eggs within the oviduct, the lumen of which is conspicuous in the loops already traversed by the eggs and also in the adjacent loop into which the ova will soon pass. No cilia are found in the isthmus.

The last section of the oviduct is rightly called the intramural region or the tubo-uterine junction, since it passes into and through the upper wall of the uterus. Instead

of ending flush with the endometrium, however, the tube continues into the cavity of the uterus as a blunt papilla, the colliculus tubarius (see figures 3 and 6). Figure 6 is of particular interest because it shows the anatomical relationship of the tube to the uterus. Even the ostium on the tubal papilla may be distinguished. Normally, before the tube is straightened, the papilla more closely approximates the ovarian end of the uterus because the tube enters at an angle. This intramural region is also without cilia.

Transportation of Ova Through the Fallopian Tube

Expulsion of ova from the ampulla. When first seen in the second loop of the ampulla, the eggs are closely surrounded by sticky cumulus cells, and are accompanied by

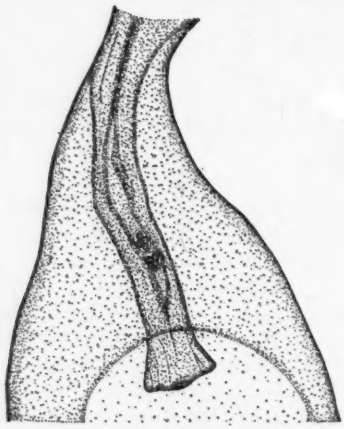


Fig. 3. TRACING FROM A PROJECTION OF THE TRACT SHOWN IN FIGURE 9, TO SHOW THE POSITION OF THE TWO OVA IN RELATION TO THE WHOLE INTRAMURAL REGION.

fluid from the periovarial sac. Frequently, the ampulla is so distended with the egg mass and fluid that even superficial examination shows that ovulation has taken place, and by means of proper lighting the individual eggs can be distinguished. Such a condition is shown in the paper negative, figure 4. The cilia on the longitudinal folds, which appear as white stripes in this negative, are particularly effective at the time of ovulation and for a short period after the eggs reach the ampulla. Although we have never seen eggs actually enter the ampulla, we assume that the cilia play an important rôle in sweeping the eggs into the oviduct and carrying them to the second loop. Recent observations following induced ovulation (6) show that ciliary action in this second loop is sufficiently strong to rotate the whole cluster of eggs. One typical observation showed the egg cluster being carried ad-ovarially along the inner side of the mesosalpinx, then ab-ovarially along the outer surface, the whole rotation taking 20 to 25 seconds. This action, however, is apparently only temporary, ceasing shortly after the eggs reach the ampulla. Although the cilia continue to circulate small particles, such as dead sperm cells and leucocytes, they become ineffective in moving the egg mass. Whenever ova, devoid of cumulus cells were experimentally pressed from the isthmus into the ampulla, only peristalsis was involved in returning the eggs to the isthmus.

After the egg cluster comes to rest in the second loop, the ampulla becomes par-

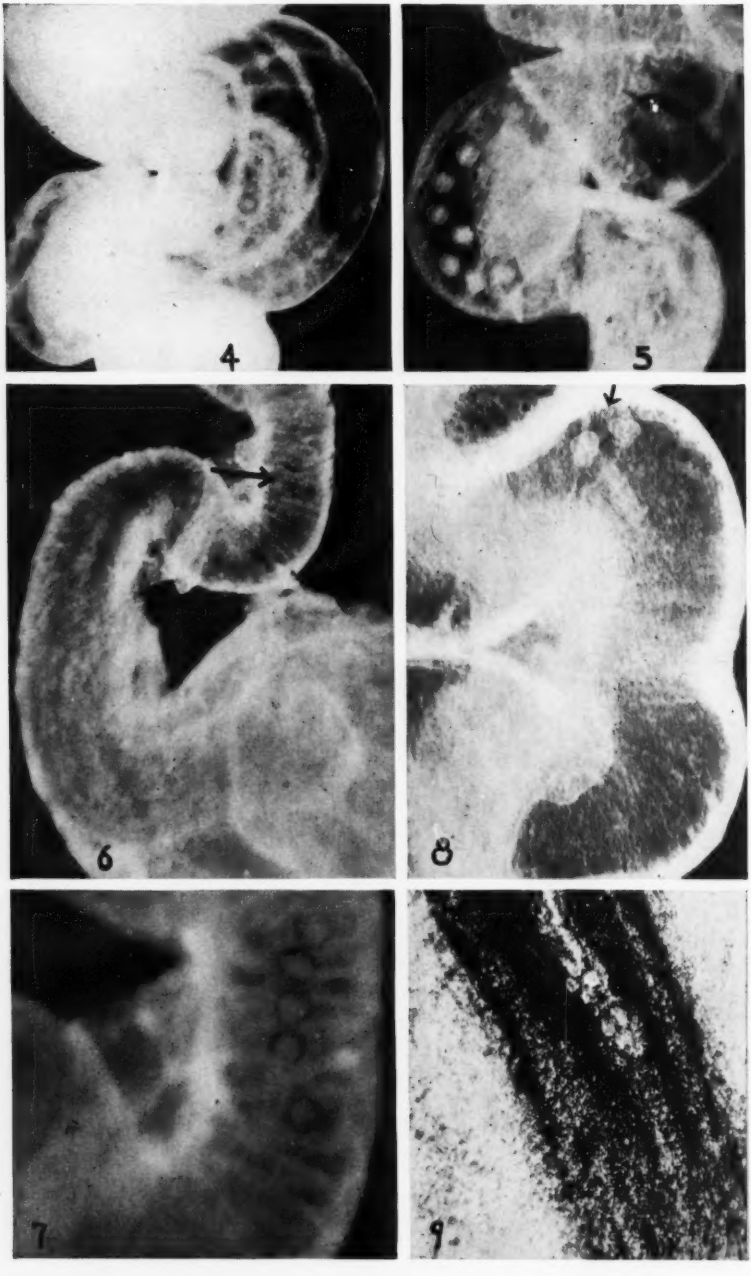
ticularly quiet. Even the isthmus is relaxed and its lumen more open than it is later, when contractions begin in the ampulla. We attribute this quiescence and relaxation of the tubal musculature, in part, to the effect of seminal fluid, because the uterus is always quiet and flaccid after having been filled for several hours with this material, although before ovulation the uterine muscles are activated by estrogen (7, 8, 9). The tube recovers its muscular tone earlier than does the uterus, possibly because it has been exposed to less semen. Before this recovery, the ascent of sperm cells is possible, but the eggs themselves are excluded from the isthmus by the size of the cumulus mass.

After fertilization, the cumulus cells begin to fall away from the ova, in which action peristalsis is apparently an accelerating factor. One typical observation showed moderate contractions of the ampulla occurring at intervals of 5 seconds, yet cumulus cells were still present 12 hours after the vaginal plug was found. However, the egg mass was not so compact and opaque as at first, and individual eggs were more easily seen. In another specimen (fig. 5) about 20 hours after the vaginal plug, the eggs were free of cumulus cells and local peristalsis had moved the ova forward to the entrance of the isthmus. These peristaltic waves were spaced 12 to 16 seconds apart and were in definite sequence: first, a gentle, ad-ovarial contraction forced the fluid and eggs approximately a half-loop up the tube; then a considerably stronger ad-uterine peristaltic wave rolled and tumbled the ova down to their former position. Meanwhile, the adjacent, lower half-loop of the isthmus gradually expanded so that the ova were eventually forced into that section.

In this back-and-forth movement of the eggs there is a reciprocal relationship between the two ends of the loop holding the eggs. That is, the circular muscles relax at the ovarian end of the loop so as to accommodate the contents of the tube which are forced there by the ad-ovarial contraction. This sudden increase in bulk evidently stimulates the muscle fibers for they immediately contract vigorously, propelling the eggs back to the uterine end of the loop. All of these contractions appear to be typical smooth-muscle responses.

The question frequently arises as to the length of time that cumulus cells remain attached to the ova. Lewis and Wright (10) indicate that these cells are lost about 12 hours after ovulation, although we have found them present 15 hours after the vaginal plug. Pincus (11) has shown that dispersion of cumulus cells soon takes place in the presence of seminal fluid *in vitro* and that the speed of dispersion is roughly proportional to the concentration of the sperm emulsion. We agree with Lewis and Wright that, *in vivo*, unfertilized ova of unmated mice are also denuded of cumulus cells in the ampulla, so that they, like fertilized ova, may be found entering the isthmus approximately 24 hours after ovulation. If, then, neither fertilization nor the presence of seminal fluid is entirely responsible for the denudation of eggs, we must look to other local chemical changes and to peristalsis as possible factors.

Peristalsis in the isthmus. We were often impressed by the fact that the tube is rather quiet during the passage of eggs through the isthmus, except in the immediate vicinity of the eggs. We wish to emphasize the local nature of these peristaltic and antiperistaltic movements of the tube (see protocol) because the eggs are rolled back and forth in a single loop for some time. The contraction waves which Westman (1) saw in the winding part of the rabbit tube may also have been in sections with ova because he observed that they were generally confined to one loop (p. 88). In view of the fact that several of the earlier observers reported that the tubes were quiet during the period of tubal transport, we are led to believe that they may have overlooked the contraction waves limited to that section or sections carrying the eggs. This would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be entirely possible with mouse oviducts, in which the tight coils and mesosalpinx easily obscure such reactions. It is only when the tube is uncoiled and microscopically inspected that these contractions are apparent. A study of the nature of these movements is especially important because of the absence of cilia in the isthmus of the fallopian tube of the mouse.

There have been numerous opportunities to study the activity of the oviduct while eggs were in the isthmus. One of these observations lasted over three hours, during which time the 4-celled eggs, in two different regions of the tube, descended into lower loops. We have included the protocol and an outline drawing (fig. 2) of this tract. Section numbers in the diagram do not indicate true loop numbers, but are included for descriptive purposes.

PROTOCOL

Animal killed at 10 P.M. Uterus quiet in body cavity; left tract removed for observation. Activity observed in *Section VI*. With light properly adjusted, 4 ova were found being rolled about in the lumen by peristaltic and anti-peristaltic waves which were regularly spaced 5 seconds apart; lumen apparently open and full of liquid.

Section IV was equally active. This was not expected, because greatest activity had formerly been found only in the region with ova; however, when *Section IV* was carefully examined, 2 more ova were found there. This fact supports previous observations of localized activity, especially since *Section V* showed only an occasional slight contraction. *Sections I, II,* and *VIII* were very quiet. Only slight activity was seen in *Sections III* and *VII*.

Contraction waves in *Section IV* did not proceed through to the uterus. Moreover, sectional peristalis appeared to be limited to the half-loop portions which enclosed the ova. Adovarial contractions did not force ova back into adjacent anterior loop because the contractions were confined to the loop with ova. Many times it seemed that the ova should certainly be forced down into the next loop, but the contraction waves stopped and anti-peristalsis carried the ova to the ovarial end of the half-loop. There was no evidence of ciliary activity.

At 11:40 P.M. the 4 ova in *Section VI* were finally washed around the curve into *Section VII*. Later, *Section IV* repeated this whole process and the 2 ova descended to *Section V*. Activity continued for at least one-half hour in sections abandoned by the ova, but at the end of this period their activity was greatly lessened. On the other hand, *Section V* and *VII*, now with ova, had by this time acquired greater activity. This was particularly true of *Section VII* where contraction waves were about one second apart.

At the end of three hours of observation the 4 ova were still in *Section VII*. The lumen in the curve of *Section VIII* was opened somewhat so that the movements of debris in fluid were seen. Lazy contractions of the uterus were noted throughout this period, but none of them altered the position of ova.

Fig. 4. PHOTOMICROGRAPH OF AN AMPULLA SHOWING EGGS SURROUNDED BY CUMULUS CELLS. The isthmus is the white mass below the eggs. ×20. Fig. 5. PHOTOGRAPH OF THE LOWEST LOOP OF AN AMPULLA CONTAINING EGGS THAT ARE DENUDED OF CUMULUS CELLS and are about to enter the narrower muscular isthmus. This ampulla was unusually distended with fluid. ×45.

Fig. 6. PHOTOMICROGRAPH OF THE LOWER ISTHMUS AND INTRAMURAL REGION. The arrow points to eggs in the isthmus. Of special interest is the shadow of the tubal papilla extending into the uterine lumen which is also shown in outline. ×20.

Fig. 7. PHOTOMICROGRAPH OF THE ISTHMUS AND EGGS SHOWN IN FIGURE 6. This tube was less compressed by the cover glass than that shown in figure 8. ×60.

Fig. 8. PHOTOMICROGRAPH OF EGGS IN THE ISTHMUS. Arrow points to 2 eggs, both of which are in the 4-celled stage. The diameter of the lumen is exaggerated due to pressure on the cover glass. Note that the zonas, which are always present at this stage, do not show through the tubal tissue. ×60.

Fig. 9. PHOTOMICROGRAPH (POSITIVE) SHOWING TWO OVA IN THE INTRAMURAL REGION of the oviduct 50 hours after the vaginal plug was found. The lumen is expanded above the eggs, but is open only a short distance below them. Compare with figure 3. ×80.

Did Locke's solution stimulate the tube to an unusual degree of peristalsis? Probably not, because all regions would be similarly affected. As it is, empty loops remained relatively quiet, while those with ova were active.

It normally takes 20 to 24 hours for eggs to travel the length of the isthmus. In this journey the eggs are always found in the central portion of the lumen. We have never seen them in the crevices made by folds of tubal epithelium. Although eggs are not so visible in this region as they are in the ampulla, individual blastomeres can be seen when slight pressure is applied to the cover glass. The zona pellucida, however, remains invisible. Such a condition is shown in figure 8 with two 4-celled ova. In the tube not flattened by pressure, ova appear as in figures 6 and 7 which are photographs of the same tract under different magnifications.

Transport through the intramural region. The factors controlling the transportation of eggs through the intramural region are not yet clear. The eggs are usually in this area on the third day after fertilization, and their progress through the relatively short portion is slow, requiring approximately 24 hours. The lumen is much narrower here than it is in the isthmus, and the fluid in the tube is much reduced. The eggs seem to be pushed along as the lumen widens before them. Figure 9 is a photograph of two ova in this region. It will be seen that the lumen is open in the immediate vicinity of the eggs, but it is not sufficiently relaxed to give free passageway to the uterus. The diagram, figure 3, which was traced from a projection of this tract, indicates the position of these eggs in relation to the papilla and uterus.

What part, if any, uterine muscles play in the transport of eggs through this region is not known. However, peristaltic activity in the uterus and tubo-uterine region is greatly increased on the third day, so that probably the combined efforts of these muscles propel the ova past the relaxed sphincter into the uterus.

DISCUSSION

It is interesting to know that Squier (12) reported that both Bischoff in 1852 and Spee in 1883 also saw the tubal ova when using transmitted light Squier observed ova in the tube of the guinea pig under similar conditions, and his comments on tubal activity are worth quoting (p. 232). ". . . the tube, which has been kept in salt solution at body temperature, spontaneously contracted and relaxed lazily; and as it did so, the egg within it shifted to and fro with perfect ease, certainly without any suggestion that it was sticky or that any obstruction in either direction was offered by the cilia or anything else in the tube. It behaved like a free body in water." Our studies confirm this casual observation in so far as conditions in the ampulla and isthmus are concerned.

If peristalsis is responsible for egg transport, then questions arise concerning the factors which initiate local contractions, especially when the eggs are in the ampulla. The stimulative agent would appear to come from the contents of the tube, but the source cannot be the developing embryos, because we have found, as have Lewis and Wright, that non-fertile eggs pass along the tube at about the same rate as do the fertilized ova. It is possible that either the follicular liquor, introduced into the ampulla at ovulation, or the cumulus cells may be the source of the necessary stimulant. Since this exciting agent apparently becomes effective as the cumulus cells begin to disintegrate, these cells may furnish either estrogen or electrolytes capable of locally activating the smooth muscle fibers. It might seem that the new corpora lutea have by this time begun to form estrogen in amounts sufficient to stimulate the isthmus, were it not for the fact that the ova of animals ovariectomized after mating also pass to the lower portion of the oviduct. Corner (13) showed that the ova of rabbits öophorecto-

mized 14 to 18 hours after mating, pass into the uterus. Whether or not their entrance into the uterus was delayed was not indicated, as his animals were autopsied 4½ to 7¾ days after mating. We have reported that the eggs of mice ovariectomized within 12 hours after mating are often delayed in their exit from the oviducts, but that they may be accelerated in their passage by injecting adequate amounts of an estrogen (14). This may indicate that, although the chemicals within the oviduct are sufficient to propel the ova into the uterus, their action is normally strengthened by extra-tubal factors so that the entrance of the eggs into the uterus is accomplished in approximately 3 days.

At this point one may again speculate on the reasons why the tract, including the uterus, is so quiet after copulation. We have indicated a possible effect of chemicals in the seminal fluid. However, Reynolds and Friedman (15) reported a marked decrease in uterine activity in the mated rabbit, even before ovulation. Later, (16) these investigators showed a similar reduction in uterine motility within 8 hours after an injection of pregnancy urine. Therefore, Reynolds (16) suggests that this quiescence may be due to a postcoital inhibitory factor produced by the pituitary. Whatever the cause of this reduced activity commencing about the time of ovulation, it is apparent that the ampulla is the first to recover and begin definite peristaltic waves which finally force the ova into the isthmus about 24 hours post coitum.

On the third day, in normal animals, increased activity of the tubo-uterine region and the uterus is possibly the result of the increased hormone production by the corpora lutea under the stimulus of pituitary substances. This conclusion is supported by the fact that the inhibition of the gonadotropic action of the pituitary on the corpora lutea by small amounts of estrogen, results in the retention of ova in the oviducts (17). Furthermore, massive injections of an estrogen accelerate the rate of tubal passage (18). It is thought that this large amount has a direct effect on the oviduct, sensitizing the whole tract from end to end so that the usual slower local priming of muscles is obviated. It may also act in combination with estrogen from the corpora lutea, since massive injections of an estrogen over a short period enhance their growth (19, 20, 21).

It is apparent that bulk in the form of fluid or eggs is sufficient to excite the muscle fibers if they have been previously primed by the stimulating factor. This would account for the peristalsis being limited to those sections with ova. One common observation is pertinent: whenever eggs were squeezed back into the quiet anterior loop, that region resumed its peristaltic movements. Evidently the return of the eggs and fluid quickly incited contractions in that loop which had only recently emptied itself. Wislocki and Snyder's experiment (22), in which eggs of an induced ovulation were rapidly transported through the tubes following the fertilized eggs, may be a case in point. One may infer that the tubal fibers, already primed by the passing of the first cluster of eggs, responded by rapidly propelling the second group. This contraction-response of smooth muscles to increased bulk is similar to that found in the intestines and in the uterus distended with a foreign body (23).

It would appear, then, that the ruptured follicles pass to the oviduct sufficient amounts of substances necessary for the transportation of eggs through the isthmus during the first two days. On the third day, the eggs are in the intramural region where they make slow progress. Meanwhile the corpora lutea are increasing the production of hormones necessary to bring about a greater relaxation of the tubo-uterine junction, allowing the descent of ova into the uterine lumen. This may well be the physiological explanation for the three-day journey of ova through the fallopian tubes. Furthermore, these direct observations definitely support Corner's earlier deduction

(24) that the transportation of ova through the fallopian tubes is dependent upon muscular activity rather than upon ciliary action.

SUMMARY

Under proper lighting conditions, ova can be seen inside the fallopian tubes of albino mice. Direct observations show that peristalsis is responsible for the transportation of the eggs from the ampulla to the uterus. Cilia, however, may play an important rôle in getting the eggs from the periovarial sac to the ampulla. Although eggs were never seen to descend into the ampulla, ciliary action in this region is sufficiently strong to rotate, *en masse*, several eggs with their cumulus cells. This action is apparently only temporary, ceasing shortly after the eggs reach the ampulla.

The ampullar walls are quiet until after fertilization when gentle contractions roll the eggs about, a motion which apparently aids in separating them from their cumulus cells. Approximately 24 hours after ovulation, the cumulus cells having dropped away, the isthmian lumen is expanded sufficiently to allow stronger ampullar contractions to force the eggs and some of the accompanying fluid into the first loop of the isthmus. This fluid seems to stimulate the muscle fibers. True peristalsis is confined largely to those loops in the immediate vicinity of the eggs, and appears to be a typical smooth-muscle response to increased bulk. The eggs are slowly advanced as each successive loop becomes sensitized and contractile. Transportation through the isthmus requires only about 24 hours. The eggs are slowly advanced in the intramural region and may be found in the uterus at the end of another 24-hour period.

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EFFECT OF VITAMIN B COMPLEX DEFICIENCY ON INACTIVATION OF ESTRONE IN THE LIVER

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IN A PRELIMINARY NOTE (1) we have reported that deficiency of the vitamin B complex in female rats markedly diminishes the inactivation of estrone in the liver. Further study of this phenomenon, which is the subject of the present communication, shows that the inactivation mechanism, impaired by vitamin B deficiency, may be restored by addition of brewers yeast to the diet. Subsequent depletion of the diet is again capable of interfering with the destruction of estrone in the liver. The flow of estrogen through this organ can thus be controlled at will, by withholding the vitamin B complex or by restoring it to the diet.

Within a few years after the isolation of the first crystalline estrogen, it became apparent that this and related compounds are rapidly inactivated in the body. Much of the early literature has been reviewed elsewhere (2). Perhaps the earliest clue to the possibility that estrogen is destroyed by an organ or organs in the portal circulation was the observation by Evans and Burr in 1926 (3) that estrogen was more effective when given subcutaneously than when administered intraperitoneally. The first report that the liver might be a site of inactivation appears to have been that of Silberstein, Molnar and Engel (4). However, as Zondek (5) has pointed out, the method employed by these workers was not adequate; they also reported that estrogen was destroyed by dog's blood, a finding that neither Zondek nor Israel, Meranze and Johnston (6) were able to confirm. It was Zondek (5, 7) who first reported an extensive investigation of this problem; he demonstrated conclusively that the liver is capable of inactivating from 80 to 90 per cent of added estrogen *in vitro*. Israel, Meranze and Johnston (6) showed that estrone was not inactivated when circulated through a heart-lung perfusion system; it was rapidly inactivated by perfusion through a heart-lung-liver system.

Golden and Sevringhaus (8) transplanted rat ovaries to the mesentery and to the axillae; estrus did not occur in animals with ovaries in the portal circulation but did occur in those with transplants in the axillae. Utilizing the method of pellet implantation first devised by Shelesnyak and Engle (9) and afterwards applied to crystalline steroids by Deanesly and Parkes (10), G. R. Biskind (11–15) implanted pellets of various crystalline estrogens and androgens in the spleens of castrate male and female rats. With the spleens containing the pellets in the normal situation in the portal circulation, no estrogenic or androgenic effect was obtained with estrone, estradiol, estradiol benzoate, or with the testosterone, testosterone propionate or methyl testosterone. Inactivation of both estrogens and androgens occurred in the livers of male and female rats. If the spleen containing the pellet of steroid was subsequently transplanted subcutaneously and, after establishment of a collateral circulation, the

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pedicle was ligated, the specific estrogenic or androgenic effect became evident. These observations served as the basis for the investigation reported here.

Talbot (16) extended the observation of Golden and Sevringhaus on destruction of endogenous estrogen by demonstrating that a liver poison, carbon tetrachloride, can impair the estrogen-inactivating mechanism. Administration of carbon tetrachloride to rats from 21 to 25 days old with intact ovaries led to a definite increase in the weight of the uterus over that of control animals. This effect did not occur in animals previously castrated. Pincus and Martin (17) confirmed the observation that administration of carbon tetrachloride impairs the estrogen-inactivating system; the effectiveness of a given dose of estrone was thus increased about 80 per cent.

In a fundamental *in vitro* study on the mechanism of inactivation of estrogens, Heller (18) found that in the rat, the liver converts estrone to a much more active substance, presumably estradiol, if the inactivating system is first inhibited with sodium cyanide. Thus it appears that in this animal, the liver first converts estrone to estradiol and then destroys it, and that two different enzyme systems are implicated.

Knowledge of the rôle of the liver in the inactivation of certain steroids was considered important mainly from the standpoint of therapy with these substances, especially by the oral route. But in 1940 Glass, Edmondson and Soll (19) reported that in male patients with cirrhosis of the liver, the estrogen in the urine appeared in active (unconjugated) form. Gynecomastia and testicular atrophy were associated with this excess of free estrogen. This, together with the observation of Talbot, was the first clue that the liver has an important part in maintaining the normal estrogen level of the body.

Numerous studies (20–25) have now established that cirrhosis of the liver can be produced by nutritional deficiency or nutritional imbalance and that the B vitamins (especially choline) and the protein content of the diet play a major rôle in this phenomenon. In addition to the lesion just mentioned, other disturbances have been produced in the liver, both functional and morphologic, by vitamin B deficiency (26, 27). György and Goldblatt (20, 21) have shown that in experimental cirrhosis of the liver in rats, the lesions may be prevented by the administration of brewers yeast, yeast extract or choline. Subsequently, Blumberg and McCollum (28) also reported prevention of dietary cirrhosis with choline and Lowry and his co-workers (29) have reported successful treatment of this condition with choline and casein. Patek (30) and Patek and Post (31) have noted amelioration of clinical hepatic cirrhosis with dietary therapy, mainly with sources of the vitamin B complex. Choline may also be useful in clinical cirrhosis (32).

It has been demonstrated that the lesions produced by certain liver poisons resemble, at least in part, those which occur in nutritional deficiency (20). Liver damage produced by carbon tetrachloride or chloroform may be prevented by liver extract (33, 34); that induced by lead arsenate may be prevented by brewers yeast (35). Cirrhosis and carcinoma of the liver caused by dimethylaminoazobenzene ('butter yellow') may be prevented, in part or entirely, by liver extract, brewers yeast, yeast extract, rice bran extract or by riboflavin and casein (36–40). Rhoads (41) has pointed out that intoxication with butter yellow produces a secondary or 'conditioned' deficiency of factors of the B complex. Rhoads and his co-workers have shown that breakdown products of butter yellow inhibit the cozymase system in very minute amounts.

Thus it appears probable from the evidence cited and that presented in this paper, that the impairment of the estrogen-inactivating mechanism in the liver which results from poisoning with carbon tetrachloride is really dependent on a secondarily-induced vitamin B deficiency.

EXPERIMENTAL PROCEDURE

Pellets of estrone were implanted in the spleens of a series of previously castrated adult female rats of the Sherman strain. After a brief interval to permit recovery from the operation, daily vaginal smears were made.

Eleven animals remained anestrous. The others, approximately equal in number, were found either to be in constant estrus or to have irregular estrual cycles. These animals were killed and at necropsy all were found to have one or more adhesions to the spleen or to some other organ in the portal system that would permit blood from the spleen to flow into systemic vessels without first passing through the liver.

TABLE 1. BODY WEIGHT OF CASTRATE RATS WITH SPLENIC ESTRONE IMPLANTS ON VARIOUS DIETS.

Rat No.	Body Weight, Grams, During Experiment					Average Amount of Estrone Absorbed Per Day
	At implantation	B-free diet begun	Yeast added	B-free diet begun	End of experiment	
						μg
1	272	248			136	10.0
2	268	246			162	7.3
3	268	264			156	7.3
4	235	248	152		262	13.0
5	214	220	140		249	14.0
6	219	235	115		215	16.0
7	228	253	217	254	210	13.8
8	220	211	124	235	106	14.4
9	240	225	155	260	169	12.3
10	216	244	184	247	200	14.0
11	253	265	202	264	214	13.0

The 11 anestrous animals had weighed from 214 to 272 gm. at the time of implantation of the pellets. Subsequent body weights are given in table 1. The pellets of estrone implanted in these animals weighed from 4.38 to 5.89 mg.

After control periods of from 11 to 21 days, during which the animals had no estrual reactions, they were placed on a vitamin B complex-free diet.[1] Daily vaginal smears were continued. In three of the animals estrual reactions were detected as early as the second day of dietary depletion; in others the first reaction occurred in from 11 to as long as 23 days. Usually irregular reactions occurred at first to be followed later by continuous estrus. After varying periods of estrus, indicated in figure 1, 25 per cent by weight of brewers yeast[2] was added to the B-free diet of all but three of the animals. Within from 4 to 6 days all of the rats receiving the yeast became anestrous and remained so (in one case this response occurred in 2 days). After a variable period on the brewers yeast, 5 of the animals were again placed on the vitamin B complex-free diet and once again all 5 animals became estrous.

All of the animals were killed at the end of the experiment and the contents of the peritoneal cavity were carefully examined. In 10 of the rats no adhesions of any kind were found in the abdomen that might have permitted blood from the spleen to enter the systemic circulation directly. In one animal (*rat 4*, fig. 1) a very thin adhesion was found between the omentum and one laparotomy scar. A small amount of splenic blood may thus have by-passed the liver, but this amount was not sufficient to interfere with the phenomenon observed.

[1] Vitamin B complex-free diet (Smaco): sucrose, 68 per cent; vitamin-free casein, 18 per cent; vegetable oil, 8 per cent; USP salt mixture No. 2, 4 per cent; USP cod liver oil, 2 per cent.

[2] Anheuser Busch strain 'G' containing approximately 0.15 mg. of thiamin and 0.07 mg. of riboflavin per gm.

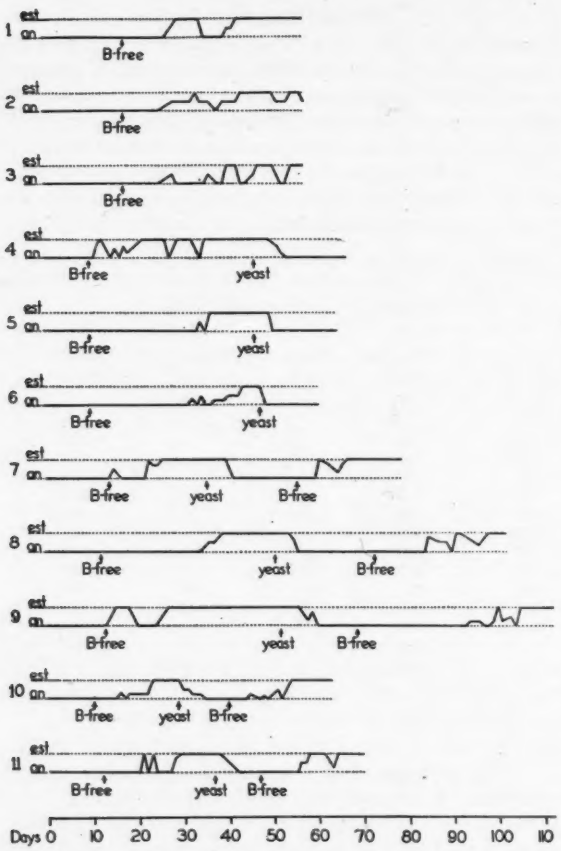

Fig. 1. Estrous reactions of castrate rats with splenic estrone implants on B-free and supplemented diets. *est:* estrus; *an:* anestrus. Smears having cellular elements intermediate between those found in anestrus and in full estrus were obtained in the transition stages of these experiments. These are shown in the chart in positions indicating the estimated proportions of the different cells present, but it is of course recognized that they are not strictly of quantitative significance.

The pellets were removed from the spleens; dried for several days and weighed on a torsion micro-balance. The average amounts of estrone absorbed per day varied from 7.3 to 16 micrograms. (See table 1.)

The livers of these animals all appeared normal grossly. Sections of liver from each animal were examined microscopically.[3] The liver of *rat 11* (fig. 1) showed several small, scattered, focal collections of mononuclear cells with a few polymorphonuclear cells and some destruction of liver cells in these areas (this rat had twice been depleted in the B vitamins). No significant changes were seen in any of the other livers; the liver cells and Kupffer cells were normal in appearance in all of them.

[3] We are indebted to Dr. R. J. Lebowich of the Eugene Littauer Memorial Laboratory, Gloversville, N. Y., for his kindness in preparing these sections with his soap-wax vacuum impregnation technic.

DISCUSSION

In a corollary investigation, M. S. Biskind and Shelesnyak (42) have demonstrated that the estrogen-inactivating mechanism of the liver may be impaired at a stage of vitamin B complex deficiency when the ovary is still functional. They castrated a series of female rats and transplanted one ovary to the spleen. On a normal diet, animals that had no adhesions to organs in the portal circulation that might have permitted blood from the spleen to by-pass the liver, remained anestrous. When these animals were placed on a vitamin B complex-free diet, estrual reactions occurred.

It appears from the evidence already cited and from that presented here, that the liver may have an important part in maintaining the estrogen level of the body within the normal range. This applies not only to estrogen secreted in the body but probably also to that consumed in the diet.[4] Failure to inactivate a portion of the estrogen normally secreted in the body and failure to destroy dietary estrogen as well, may be responsible for disorders associated with a high level of body estrogen, such as menorrhagia and metrorrhagia, uterine and mammary hyperplasias and neoplasias, premenstrual tension, some cases of dysmenorrhea, depression of anterior pituitary function and other secondary disturbances. As already mentioned, Glass and his coworkers (19) have demonstrated this relationship in the case of testicular atrophy and gynecomastia in the male.[5] The great variability in effectiveness of estrogen administered by mouth for therapeutic purposes may also be explained on this basis.

A severe degree of vitamin B deficiency is not necessary for impairment of the hepatic estrogen-inactivating function. In the experiments reported here, estrus occurred from the splenic estrogen implants in some animals at a stage of B complex deficiency when less than 9 or 10 per cent of the body weight had been lost.

The occurrence of menorrhagia and metrorrhagia, early in the disease, in women with cirrhosis of the liver has long been known; reference to this fact is made in Osler's Modern Medicine, published in 1908 (43). Menstrual disturbances have been reported to occur in intoxications with liver poisons, such as lead (44, 45), benzene (46, 47) and carbon disulfide (44). Bleeding produced by the latter two substances may of course be referable to changes in the blood and the rôle of the liver would thus be difficult to evaluate. Little attention has been paid to this subject by most of the writers on industrial hygiene. The menstrual history is usually ignored. A more comprehensive study of the literature on the toxicology of liver poisons and an investigation of clinical histories and necropsy reports is now under way.

SUMMARY

Pellets of estrone were implanted in the spleens of adult castrate female rats; the absorbed estrogen must pass through the liver before reaching the systemic circulation. On a normal diet the animals remained anestrous. When these rats were placed

[4] In a study concerned with possible physiologic functions of dietary estrogens and other substances, Casimir Funk and I. C. Funk (*Science* 90: 443. 1939) investigated the effect of orally administered estrogen on rats receiving complete and partially deficient diets. When estrogen was given by mouth to normal animals on a substantially complete diet, there was no significant effect on uterine weight. After 26 or 34 days on a diet that was probably partially deficient in factors of the vitamin B complex the uterine weight was markedly diminished. Oral administration of estrogen to rats on this diet led to increases in uterine weight of about 100 per cent. This study might be taken to indicate that the liver failed to inactivate estrogen in the B-deficient animals. Unfortunately, however, the data presented neither support nor refute this contention, owing to the fact that the dosages of estrogen employed in the animals on the deficient diet were 50 per cent and 350 per cent greater than those given the control animals.

[5] The loss of pubic, axillary and body hair that occurs in male patients with cirrhosis of the liver may likewise be related to the presence of excess estrogen.

on a vitamin B complex-free diet, protracted estrus occurred. Addition of brewers yeast to the diet caused the animals to become anestrous. Subsequent dietary depletion again led to estrus. Except in one instance no significant lesions were found in the livers of these animals. The possible clinical significance of these findings is discussed.

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IMMUNOLOGICAL IDENTITY OF INSULIN FROM VARIOUS SPECIES[1]

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THE STRIKING similarity in the physiological activity and in the chemical analysis (1) of purified extracts of insulin derived from various animal sources prompted further work in an attempt to demonstrate more fully the identity of insulin prepared from different species. An immunological approach has been used since it already has been demonstrated that insulin is active as an antigen, producing complement fixation antibodies (2) and anaphylactic antibodies (3). Using the Schultz-Dale anaphylactic response, Lewis (3) demonstrated that typical reactions indicative of anaphylaxis may be produced by sensitizing pigs with insulin extracted from the pancreas of one species and then using, as the shocking antigen, insulin produced from an entirely different species. The report of Bernstein, Kirsner, and Turner (4) on anaphylaxis in guinea pigs, using insulin as the antigen, is scarcely decisive in settling the point in question since the same type of insulin was used both for sensitization and for shocking purposes.

An indication that insulin *per se* is the same at least immunologically, no matter what the source, was seen in previously reported work on the complement fixation response with insulin as an antigen (2). In this report further confirmation of the immunological identity of insulin will be presented.

METHOD

The methods for immunizing animals has been described in a previous report (2). A greater variety of insulin preparations was used in the present investigation. The majority of the animals was sensitized to beef insulin. As the complement fixing antibody, beef, pork, sheep,[2] bison,[2] dog and human insulins were used. The latter two were prepared according to the method of Scott and Fisher (5).

In a number of instances, sensitization was produced with inactivated insulin since it has been shown that the antibody response is apparently unimpaired when insulin is inactivated with cysteine. In this way larger amounts of insulin may be used as an immunizing antigen without the necessity of administering large amounts of glucose to counteract the hypoglycemia consequent to the injection of active insulin.

Anaphylactic reactions in guinea pigs were made essentially according to the method outlined by Bernstein, Kirsner and Turner (4). The animals were immunized with a single injection of active insulin. After a sensitizing period of 21 days the animals were given a shocking dose by the intravenous injection of insulin from another species. If no reaction occurred, a second period of 21 days was allowed to pass before another shocking dose was given.

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[2] We are indebted to the Eli Lilly Co. Indianapolis, Ind., for generous supplies of insulin preparations, and to Dr. D. A. Scott, of the University of Toronto for a supply of sheep and bison insulin.

TABLE 1. ANAPHYLACTIC REACTION TO INSULIN DERIVED FROM DIFFERENT SPECIES

Guinea Pig Number	Date	Insulin	Amount of Insulin, U	Remarks
1	11/15/40	Inactivated crystalline beef	40	Intraperitoneal injection
	12/ 7/40	No injection		
	12/28/40	Active pork	20	Intravenous injection; no reaction
2	11/15/40	Inactivated crystalline beef	40	Intraperitoneal injection
	12/ 7/40	Active pork	8	Intravenous injection; no reaction
	12/28/40	Active pork	20	Intravenous injection; shock in 40 seconds; ineffective gasps 2 minutes; death at 2½ minutes
3	11/15/40	Inactivated crystalline beef	40	Intraperitoneal injection
	12/ 7/40	Active pork	8	Intravenous injection; no reaction
	12/28/40	Active sheep	20	Intercardiac injection; scratching of nose 1 minute 40 seconds; no further reaction
4	11/15/40	Active amorphous beef	20	Intraperitoneal injection
	12/ 7/40	Active pork	8	Intravenous injection; no reaction
	12/28/40	Active sheep	20	Intravenous injection; prostration 45 seconds; semi-convulsive movements ments at 2 minutes; death at 5 minutes after injection Autopsy: lungs markedly expanded
5	11/15/40	Active amorphous beef	20	Intraperitoneal injection
	12/ 7/40	Active pork	8	Intravenous injection; no reaction
	12/28/40	Active sheep	20	Intercardiac injection; cough at 30 seconds; convulsion 3 minutes 30 seconds; death 5 minutes 30 seconds Autopsy: lungs markedly expanded
6	11/15/40	Active amorphous beef	20	Intraperitoneal injection
	12/ 7/40	No injection		
	12/28/40	Active pork	20	Intravenous injection; no reaction
7	1/ 6/41	Active crystalline beef	20	Intraperitoneal injection
	1/27/41	Active pork	8	Intravenous injection, negative
	2/20/41	Active sheep	8	Intravenous injection; sneezing 45 seconds; Cough 1 minute 15 seconds; convulsions with loss of position 2½ minutes; slow recovery
8	1/ 6/41	Active crystalline beef	20	Intraperitoneal injection
	1/27/41	Active pork	8	Negative—? sneezing
	2/20/41	Active sheep	8	Negative
9	1/ 6/41	Active crystalline beef	20	Intraperitoneal injection
	1/27/41	Active pork	8	Negative
	2/20/41	Active sheep	20	Cough, 1 minute; loss of position and convulsion 2½ minutes; occasional gasp with death at about 5 minutes Autopsy: lungs marked expanded
10	1/ 6/41	Active pork	20	Intraperitoneal injection
	1/27/41	Active bison	8	Negative
	2/20/41	Active beef	8	Negative
11	1/ 6/41	Active pork	20	Intraperitoneal injection
	1/27/41	Active bison	8	Intravenous injection; negative
	2/20/41	Active beef	20	Intravenous injection; negative

TABLE 1—*Continued*

Guinea Pig Number	Date	Insulin	Amount of Insulin, U	Remarks
12	1/ 6/41	Active pork	20	Intraperitoneal injection
	1/27/41	Active bison	8	Intravenous injection; negative
	2/20/41	Active beef	20	Slow collapse, death at 7 minutes Autopsy: lungs markedly expanded

RESULTS

Anaphylactic reaction. Of 12 animals injected with a sensitizing dose of insulin, 5 responded to a shocking dose of insulin from another species with typical anaphylactic reactions which began in 3 to 5 minutes and resulted in death. It may be emphasized that rapidity of onset of symptoms obviates the possibility that the reactions were due to hypoglycemia. Three animals responded with non-fatal reactions, one being quite severe. The remaining 4 animals revealed no anaphylactic response to the shocking dose. It should be noted here that in almost all instances the reaction, whether fatal or non-fatal, occurred after a second period of 21 days had elapsed. The injection after the first 21-day period apparently resulted in no anaphylactic response.

Examining the instances of positive response in detail it is noted that in *guinea pigs* 4 and 5 the sensitizing injection consisted of 20 U of active beef insulin. Twenty-one days later an injection of 8 U of active pork insulin produced no reaction. Twenty-one days after the second injection, intravenous administration of 20 U of active sheep insulin produced typical anaphylactic response with death in approximately 5 minutes. In *guinea pig* 7 the same sequence of injection was followed with no response to 8 U of active pork insulin after a 21-day period of sensitization, but with a severe though non-fatal response to intravenous injection of 8 U of active sheep insulin after

TABLE 2. COMPLEMENT FIXATION REACTION TO INSULIN DERIVED FROM DIFFERENT SPECIES

Antigen (Insulin)	Serum Antibody[1]					Remarks
	1	2	3	4	5	
Crystalline beef		+	4+	+		Insulin solution not anticomplementary
Amorphous beef			2+	2+	2+	Insulin solution not anticomplementary
Amorphous pork	+	+	4+	3+	2+	Insulin solution not anticomplementary
Amorphous bison		+	+	+	+	Insulin solution not anticomplementary
Amorphous sheep		+	4+	4+	4+	Insulin solution not anticomplementary
Dog pancreas extract		+	3+		+	0.3 cc. extract moderately anticomplementary; 0.1 cc. not anticomplementary
Human pancreas extract			4+	4+		0.05 cc. extract moderately anticomplementary; 0.025 cc. not anticomplementary

[1] Serum antibody prepared from rabbits sensitized with beef insulin. Serum 5 prepared by Dr. Paul Cannon, Dept. Pathology, University of Chicago.

1+, reaction with at least 0.1 U or 0.1 cc. extract; 2+, reaction with .01 to 0.1 U; 3+, reaction with .001 to .01 U or with .001 cc. to .01 cc. extract; 4+, reaction with less than .001 U of insulin or with less than .001 cc. extract.

the second period of sensitization. *Guinea pig* 9 responded with a fatal reaction to the injection of sheep insulin although no response to pork insulin occurred after the first shocking dose was given. *Guinea pig* 12 received an initial sensitizing dose of active pork insulin. An initial shocking dose of bison insulin produced no effect but a succeeding injection of active beef insulin 23 days later produced death in 7 minutes with a typical picture of the lungs suggestive of anaphylactic reaction. In *guinea pig* 2 a typical anaphylactic response was obtained, but since the successful shocking antigen was derived from the same species as the previously injected antigen, it is possible that the reaction may have been due to a common pork protein contaminant.

Complement fixation. Using serum prepared from rabbits immunized with beef insulin, positive complement fixation reactions were consistently obtained with crystalline beef insulin, commercially prepared pork insulin, amorphous bison insulin, and commercially prepared sheep insulin.

In addition, with three sera, extracts of dog pancreas were run for complement fixation with positive results. With two sera, extracts of human pancreas were also run giving positive complement fixation of extremely high titer. It is noted in referring to the table that positive complement fixation was obtained with the various insulin preparations, but that the units of insulin necessary to obtain this positive reaction were not at all constant between the various types of insulin or even between reactions from day to day using the same insulin. It would seem, however, that in general, the most sensitive antigen was the commercially prepared sheep insulin which consistently gave positive complement fixation with a smaller unitage of insulin than did the bison, pork or beef insulin. At the same time the anti-complementary control as regards the sheep insulin was at all times negative. In the same manner the bison insulin consistently required a greater quantity of insulin for positive fixation than did the other types of insulin. This suggests that the antigenic property has no relationship to the physiological activity of the insulin preparation, and it is in accord with the previous finding that cysteine-inactivated insulin can be successfully used to immunize a rabbit with the formation of anti-insulin antibodies.

As regards the extracts of dogs and human pancreas in both cases the extract showed slight hemolytic and slight anti-complementary property. However, the extremely high dilution (human extracts) with which positive complement fixation was obtained as opposed to the relative large amount of solution necessary for the anti-complementary reaction suggests a true positive complement fixation with these extracts.

Of interest is the fact that in a number of patients suffering from chronic hypoglycemia it has been possible to obtain at times a positive complement fixation using the patient's serum as the antigen and the prepared rabbit serum as the anti-insulin antibody. Such a reaction has not been obtained in normal bloods. Hence, it is possible that in such instances it is the excessive amount of insulin in the circulation that is responsible for the positive complement fixation.

SUMMARY

The immunological identity of insulin derived from various animal sources is demonstrated by means of the anaphylactic reaction and the complement fixation reaction.

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PANCREATIC DIABETES IN THE OWL[1,2]

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SHORTLY after the monumental observation that pancreatectomy in the dog results in the development of the syndrome of diabetes mellitus, Minkowski studied the influence of pancreatectomy in other species. In 1892 (1) he reported that pancreatectomy in the duck, chicken or pigeon does not result in glycosuria. Similar observations had been made by Langendorf in 1879 (2) and were confirmed by Weintraud in 1894 (3) and by Kausch in 1896 (4). Since that time it has been fairly well established that the domestic fowl does not develop either glycosuria or hyperglycemia on pancreatectomy, even though such animals readily develop ketosis (5).

In 1892 Minkowski studied the influence of pancreatectomy in a single hawk and did observe a marked glycosuria after the operation (1), an observation previously made by Langendorf (6). Weintraud also studied the influence of pancreatic ablation in one falcon, two buzzards and two ravens and noted the appearance of sugar for a few days in the urine of these animals (3).

In explanation of their findings, Minkowski and Weintraud presented the hypothesis that the occurrence of glycosuria following pancreatectomy is associated with the carnivorous habits of the animal. However, since that time there has been no systematic study of this possibility, other than experiments with the duck and chicken, which confirmed the previous failure to produce a permanent diabetes.

In a previous series of experiments we studied the influence of carnivorous diets on the development of diabetes in the duck, but did not obtain positive results (5, 7). In order to test the hypothesis first postulated by Minkowski, a study of carnivorous birds was started. This report is concerned with the data obtained on a carnivorous bird, the owl.

METHOD

Twenty-two horned owls (*Bubo Virginianus Virginianus*) of both sexes were studied. The owls required a period of from several days to a week after they were caught to adapt themselves to the confinement of cage life. After the adjustment, however, they readily ate the whole mice or rats which they were offered. Samples of blood were drawn from the wing veins at various intervals for the determination of the sugar and acetone body concentration by modifications of the Somogyi (8, 9) and Barnes (10) methods.

At various times both before and after the birds were depancreatized, the blood sugar response of the animals to insulin (1 U per kg.) and glucose (1.75 gm. per kg., intravenously) was studied.

Pancreatectomy was performed in the owls under sodium phenobarbital anesthesia. It was noted that whereas the duck requires from 25 to 30 mg. of sodium

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[2] Presented in partbefore the American Physiological Society, April, 1942, Boston, Mass.
[3] Leo Lehman Fellow.

phenobarbital to induce anesthesia, a much smaller dose was required by the owl. The pancreas of the owl resembles, in all gross respects, that of the duck, and microscopic examination reveals the presence of islets of Langerhans. Little difficulty was experienced in removing the gland which is quite discrete and located in the loop of the duodenum. The immediate recovery from the operation was uneventful and within 18 hours the animal ate. However, for reasons which have not been ascertained as yet, the appetite of these animals rapidly diminished so that from 3 to 6 days after pancreatectomy the animals frequently stopped eating. Attempts to force feed various

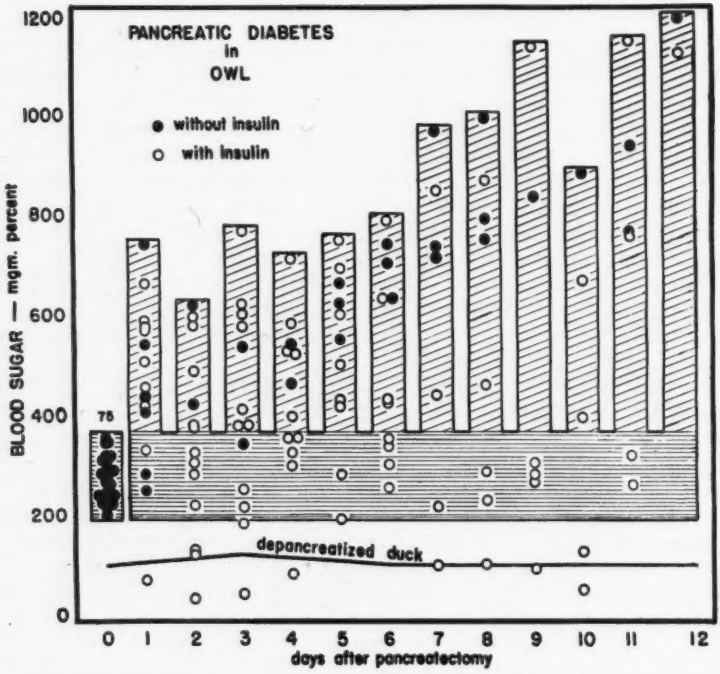

Fig. 1. INFLUENCE OF PANCREATECTOMY on the blood-sugar level of the owl. The scatter diagram depicts the findings in all of the birds studied. The solid line represents the typical response of the depancreatized duck. Approximately 1 U of insulin per kg. was given to the treated birds.

types of diet, the administration of pancreatin, beef pancreas, raw beef, or treatment with from 1 to 2 U of insulin were of no avail in preventing the loss of appetite and the subsequent death of the animal from what appeared to be inanition. Death generally occurred at the 9th to 12th day after the operation.

RESULTS

The blood-sugar concentration of the unoperated owl varied from 200 to 350 mg. per cent. It may be emphasized that the owl eats only at night and hence these blood sugars which were taken in the morning could not be regarded as the lowest possible level. However, studies performed on fasted birds did not reveal a significant difference from the above. Furthermore, in order to obviate the influence of handling and 'emotional' reactions, some owls were anesthetized and their blood-sugar level

studied for as long as 8 hours. In spite of the anesthesia, there was no significant change in the blood sugar. In view of the high initial blood-sugar levels, we regarded it of some importance to perform sugar tolerance tests and determine insulin curves on the normal owl.

It was noted that the sugar-tolerance curve of the owl is not similar to that observed in either the normal dog or duck, for in the owl a typical diabetic response is observed (fig. 1). The tolerance curve was determined by the intravenous administration of 1.75 gm. of glucose per kg. and it is quite possible that this dose of carbohydrate is too large for the performance of the test. A study of the insulin sensitivity reveals that the owl is as sensitive to insulin as are the dog, duck and other species hitherto studied (fig. 1).

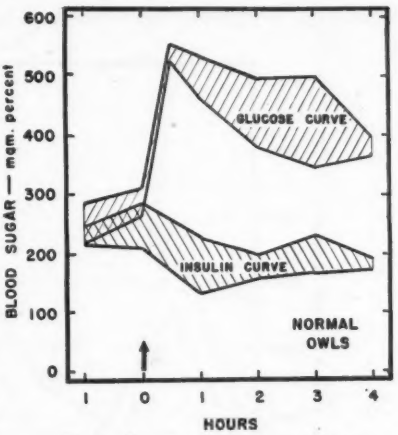

Fig. 2. Composite curves of blood-sugar response to the injection of glucose (1.75 gm. per kg.) and of insulin (1 u per kg.) at arrow.

Some of the observations made after pancreatectomy of the owl are illustrated in figure 2. It may be noted that 75 determinations of preoperative blood-sugar levels range from about 200 to 350 mg. per cent. Irrespective of whether the animal received insulin or not, an immediate rise in the blood sugar ensued to levels as high as 750 mg. during the first 24 hours, and higher thereafter. The height of the blood-sugar level could in most instances be associated with the intake of food by the animal. In the majority of instances the animals ate poorly after the third to sixth day and death rapidly ensued thereafter. Many birds died in hypoglycemia, whereas others died with a marked hyperglycemia in spite of the administration of insulin. This may be due to differences in dietary intake, since it was impossible to anticipate the amount of food that the birds would eat. For purposes of comparison, it may be noted that this is in striking contrast to the relative ineffectiveness of pancreatectomy in the duck.

Figure 3 illustrates the ability of insulin to control the blood sugar of the depancreatized owl. It may be noted that within 24 hours after the operation the blood-sugar level had risen and the administration of insulin then induced a marked fall in the concentration of the sugar. Cessation of insulin administration was followed by another rise of the blood sugar and when insulin was again administered the blood sugar again fell. When insulin administration was stopped completely, hyperglycemia and death ensued. This indicates quite clearly that insulin is effective in reducing the blood-sugar level of the depancreatized owl. However, as was indicated above, it is not effective in maintaining the animal's appetite.

Studies of the total acetone body content of the blood in these birds indicated that the concentration could be associated with the dietary intake. Animals which did not eat or ate only sparingly developed a very severe acetonemia after insulin withdrawal at the time when the blood sugar was not more than one or two hundred mg. above the initial level. On the other hand, animals which ate better did not show as severe an acetonemia.

Figure 4 depicts the typical history of an unoperated owl which was fasted in order to observe the influence of fasting itself on ketogenesis. The operated animal which had previously eaten moderately well showed a marked hyperglycemia and a more rapid rate of ketogenesis after the cessation of food and insulin than did the unoperated bird with fasting alone. Although this depancreatized bird only developed a ketonemia of 31 mg. per cent, concentrations of acetone bodies as high as 120 mg. per cent were occasionally observed in other fasted depancreatized owls.

DISCUSSION

The present studies reveal that in comparison with other species, the normal blood-sugar level of the owl is relatively high and the glucose tolerance curve is pro-

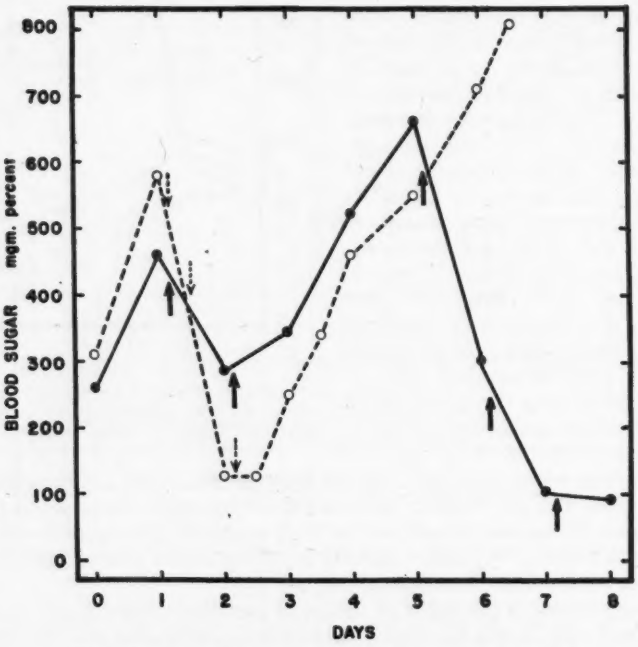

Fig. 3. Response of the blood sugar of depancreatized owls to the injection of insulin (1 u per kg.) (arrow).

longed. This does not imply necessarily that the unoperated owl is diabetic but may merely reflect the food habit of this bird, since carnivorous diets are known to similarly effect the sugar tolerance curve of mammals (11, 12).

The affect of pancreatectomy is unequivocable, but the reason that animals lose their appetite and consequently die is unknown. In a series of experiments on ducks which had been reared from the age of 1 day to 1.5 years on a diet consisting of meat supplemented with vitamins, a somewhat similar phenomenon was observed in that immediately after operation a marked hyperglycemia was noted and irrespective of whether the animal did or did not receive insulin, death ensued in 50 per cent of the animals on the third or fourth day. Those ducks which did not die showed a rapid return to a normal blood-sugar level. The preoperative blood sugar was in no instance different from that of normally fed ducks.

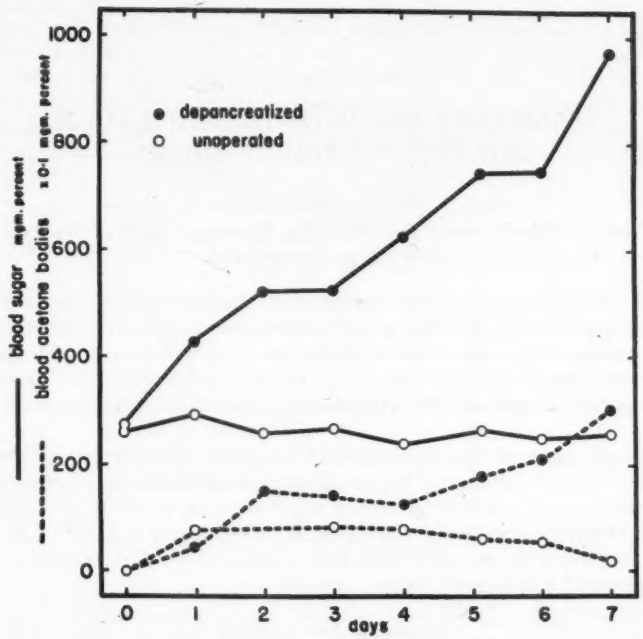

Fig. 4. Influence of fasting and of exogenous insulin deprivation on the blood sugar and blood acetone body concentrations in the normal and depancreatized owl.

It is difficult to present reasons for the development of diabetes consequent to pancreatectomy in the owl and the absence of that syndrome in the duck other than to draw attention again to the possibility that the food habits of the species may play a rôle in the susceptibility to the usual effects of pancreatectomy.

SUMMARY AND CONCLUSIONS

The blood-sugar concentration of the normal owl varies from 200 to 350 mg. per cent. The glucose tolerance curve of the normal owl is prolonged and typical of the mild diabetic state while the insulin response is similar to that observed in other species. Fasting induces the accumulation of acetone bodies in the blood.

Pancreatectomy results in hyperglycemia to levels as high as 1200 mg. per cent, a loss of appetite, an increased susceptibility to fasting ketosis and death irrespective of whether or not insulin is administered.

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MAMMARY GROWTH IN MALE MICE FED DESICCATED THYROID[1]

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THE THYROID GLAND has been considered in relation to several aspects of reproductive function. Recently several investigations have been concerned with the effects of the removal of the thyroid or addition of thyroxin, thyroglobulin or desiccated thyroid upon lactation (1, 2). Feeding desiccated thyroid to male rats has been associated with changes in the histological structure of the mammary glands (3).

During the course of experiments on the factors concerned in mammary growth in hypophysectomized male mice it became desirable to determine whether or not the concomitant general metabolic deficiency might alter the reactions of the animals. Experiments were undertaken in which desiccated thyroid was added to the diet. The diet was first tested in unoperated male mice. Unexpectedly, the mammary glands of these unoperated mice showed a marked growth.

MATERIALS AND METHODS

The mice used in this investigation were hybrid males from 40 to 60 days of age and weighing from 16 to 21.5 gm. Before the mice were selected for this experiment they were fed Purina Fox Chow (FC). To one kg. of the powdered 'Chow' 1.5 grams of desiccated thyroid[2] was added (T+FC). Other mice were fed a second commercial food (Nourishmix, N). Desiccated thyroid was added to this food in amounts of 1.5 grams per kg. (T+N). Untreated controls from the same litters were maintained on the commercial rations throughout. Food was kept before the mice continually. The animals were weighed every second day. From 17 to 49 days after the experiments were started the mice of both the control and thyroid supplemented groups were killed. The mammary glands were prepared as whole mounts of stained and dissected preparations. The testes, seminal vesicles and prostates, adrenals, livers, kidneys and thymus glands were dissected and weighed. Seven males, 4 of which received the thyroid-supplemented ration and 3 controls, were castrated at the time the experiment started.

OBSERVATIONS

Growth of the mammary glands was determined by the increase in area occupied by the branching ducts. Mammary rudiments in normal male mice are uniformly small and designated '0' in table 1. A proliferation of the ducts to an extent similar to that observed in adult virgin female mice was rated 4+ and intermediate degrees of proliferation were designated 1+, 2+, and 3+. The mammary glands of all except one

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[1] This investigation has been supported by the Jane Coffin Childs Memorial Fund for Medical Research and the Anna Fuller Fund.
[2] The desiccated thyroid (Thyroid Powder U.S.P.) was supplied by the Armour Laboratories, Chicago, Ill.

of the intact male mice fed either of the two commercial food mixtures supplemented with desiccated thyroid showed proliferation of the ducts. Mammary growth was detected in none of the castrated males and in but one of the intact mice receiving the basal diet without the thyroid. This exceptional mouse had one mammary rudiment which had attained a size equivalent to rudiments rated as 1+. The mammary glands of the thyroid-fed mice were not only enlarged but proliferation was still in progress at the time the animals were killed. Several ducts from the glands of each animal terminated in enlarged end-buds which contained cells in mitoses.

TABLE 1. TREATMENT AND MAMMARY RESPONSE OF INTACT AND CASTRATED CONTROL AND THYROID-FED MALE MICE

Number of Mice	Type of Food Consumed[1]	Period Fed, Days	Mammary Response
4	Desiccated thyroid and Fox Chow	17–38	1, 1, 2, 4
4	Desiccated thyroid and Fox Chow	17–38	0, 2, 3, 3
4	Desiccated thyroid and Fox Chow	18–39	1, 1, 2, 3
4	Fox Chow, (control)	18–39	0, 0, 0, 0
4 castrates	Desiccated thyroid and Fox Chow	18–25	0, 0, 0, 0
3 castrates	Fox Chow, (control)	18–24	0, 0, 0
2	Desiccated thyroid and Nourishmix	47	2, 3
2	Nourishmix, (control)	47	0, 1

[1] T+FC, desiccated thyroid and Fox Chow; T+N, desiccated thyroid and Nourishmix.

Each mouse gained in weight during the course of the experiments. The animals on the stock diet increased in weight the same amount as those receiving the supplement of desiccated thyroid. Although the food consumption was not determined exactly, the thyroid-fed animals ate considerably greater amounts.

TABLE 2. AVERAGE WEIGHTS OF BODIES AND SEVERAL GLANDS FROM GROUPS OF HYBRID MICE MAINTAINED ON COMMERCIAL DIETS AND DESICCATED THYROID, AND OF CONTROLS

Number of Mice	Diet	Total Body Weight, gm.	Weight of Organs, mg.				
			Testis	Kidney	Liver	Adrenal	Thymus
4	T+FC	24.7	190	514	1645	6.1	34.2
4	T+FC	26.	192	520	1785	7.5	37.3
4	T+FC	24.5	174	506	1665	6.4	36.1
4	C	24.6	200	437	1491	4.45	36.1
4 castr.	T+FC	26.5		521	2018	9.7	61.9
3 castr.	C	24.9		418	1507	4.1	61.7
2	T+N	28.8	190.3	641	2375	11.6	37.5
2	C	29.9	201.7	524	1793	4.75	27.4

The testes of the control mice weighed on the average slightly more than those of mice from the thyroid-fed groups (table 2). The range of weights of the testes of the different mice within each group exceeded the differences between the groups in most cases. The consumption of desiccated thyroid in the amounts used therefore did not alter significantly the size of the testes. Nor was there any consistent modification of microscopic structure of either the seminiferous or interstitial elements.

The weights of the seminal vesicles and prostates of the thyroid-fed mice also averaged less than those of the controls. The rather wide range in weights of these glands within each group, however, would not permit the conclusion that the differences are significant; the seminal vesicles and prostates of the castrated thyroid-fed mice were comparable to those of the controls.

The adrenal glands of the animals of all of the thyroid-fed groups averaged much larger than those from the mice of the control groups (table 2). Only two of the thyroid-fed mice had adrenal glands smaller than the largest glands of the controls. The largest adrenal glands were from the mice which had consumed the desiccated thyroid for the longest period. The glands had thickened fascicular layers and an extensive perimedullary layer of large, light-staining but well preserved cells.

The livers and kidneys of the thyroid-fed mice were on the average considerably larger than those of the controls, although the rather marked variation within the groups would not permit the conclusion that these observations are significant with the numbers of animals available.

DISCUSSION

The growth of the mammary glands in male mice fed desiccated thyroid powder mixed with their food and in amounts insufficient to influence body growth might be accounted for in several ways. The concomitant hypertrophy of the adrenals was considered as one possible contributing factor. Adrenal lesions and tumors in mice have been associated with evidence for the presence of estrogenic stimulation (4, 5). The injection of desoxycorticosterone acetate was followed by mammary growth in male mice (6). Although a striking hypertrophy of the adrenal glands occurred in the thyroid-fed castrated mice mammary growth did not occur in these animals. The hypertrophy of the adrenals was not associated consistently with the mammary response.

The failure of mammary growth in the thyroid-fed castrated males indicates that the testes are essential in eliciting this reaction. The injection of testosterone, androsterone, and dehydroandrosterone into mice was accompanied by mammary growth (6). Testicular or androgenic hormones might account for the mammary response in the thyroid-fed mice. However, if the hormones produced by the testes of the hyperthyroid mice are responsible for the mammary growth, then they must be produced in much larger amounts or altered so as to be more effective as mammotropins than testosterone appears to be.

The above possibilities are not thoroughly tenable with other observations. An increased gonad-stimulating activity of the hypophyses of experimental hyperthyroid rats has been noted by several investigators (3, 7, 8). In this respect the changes in the hypophysis resemble those which occur following castration and might indicate that the hyperthyroid state decreases the effective threshold of the gonadal hormones.

Experimentally induced hyperthyroidism in female rats was accompanied by the appearance of spontaneous pseudo-pregnancies (9). This observation might be interpreted as indicating that the pituitaries of such animals were capable of stimulating the formation and maintenance of active corpora lutea. If one assumes that the luteinizing and interstitial-cell-stimulating factors are identical, the stimulation of the testicular interstitial cells might be expected in the males. Such a stimulation could not be detected by histological examination in the testes of the mice in the present experiments.

The size of the seminal vesicles and prostates would not necessarily reveal an increased or lowered production of androgen because it is not known that these glands would afford a good indication of levels of androgen exceeding effective thresholds.

The kidneys might also indicate the level of androgen in mice (9, 10). The average renal weights of all the groups of thyroid-fed mice exceeded those of the controls. Since this was also true of the castrated mice the larger renal size cannot be attributed to an increased production of testicular androgens.

SUMMARY

The mammary glands of intact male mice fed desiccated thyroid mixed with their food (1.5 mg. per kg.) showed proliferation of the ducts and hyperplastic end buds. Castrated male mice similarily treated showed no mammary growth. The adrenal glands of the thyroid-fed, intact or castrated male mice were greatly increased in size and the kidneys and livers were also larger than in the controls. The amounts of desiccated thyroid consumed were insufficient to reduce the increase in body weight below that of the controls.

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THE REACTION OF THE CHICK THYROID TO FROG AND MOUSE ANTERIOR PITUITARIES

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IT IS WELL ESTABLISHED that the guinea pig is the best mammal to use in tests of the thyrotropic potency of the anterior lobe of the pituitary gland because its thyroid responds quickly and markedly to administration of anterior pituitary substance of many animals (1, 2). However, there is considerable evidence that the thyroid of a bird, the very young chick, is an even more sensitive indicator of the presence of thyrotropic hormone (3–8) both because the histological picture in the untreated animal is a more uniform one and therefore better adapted to reveal stimulation in large or small degree and because it responds to a smaller quantity of substance. It is also a less expensive and more easily cared for host than the guinea pig.

In some experiments in which the chick has been used the degree of the reaction has been judged mainly on the basis of changes in absolute weight in the thyroids of anterior pituitary-treated birds compared with their controls (3–7, 9). In others a qualitative histological change or a quantitative one,—the percentage increase in the height of the follicular epithelium,—in the experimental animal's thyroid compared with that in the control has been employed (8, 10–15). In still others, the decrease in thyroid-iodine and increase in blood-iodine after anterior pituitary administration has been determined and compared with that in untreated birds (7, 10, 16).

In only a few of these tests has any unit been defined. Smelser (3) emphasizes the doubling of the absolute weight of the thyroid as the effect of one unit of thyrotropic hormone. With 5 daily injections of a partially purified extract of beef anterior pituitary injected into 1-day-old chicks, which brought the total dose to 1.2 mg., the thyroid weight was more than doubled. In fact doubling of the thyroid weight was almost caused by the injection of 0.4 mg. of his extract. The unit of Kabac and Liapin (5), based on a 5-day treatment with autopsy one day after the last injection, is very similar—"As an arbitrary unit of thyrotropic preparation we designate the amount which brings the weight of both thyroids of the chick to 8 mg. (on the average) after daily subcutaneous injections during five days" (p. 337); the average weight of control thyroids was nearly 4 mg. (3.8 ± 0.3 mg. for 15 chicks). Less than a 100 per cent increase has been considered a valid reaction by Bergman and Turner (6) who administered an extract of cattle anterior pituitary to 1-day chicks of both sexes weighing from 45 to 64 gm. in 4 daily subcutaneous injections. Since they claim that the thyroid of the male chick is twice as sensitive to thyrotropic hormone as that of the female, they have established a male unit which is "the total amount of hormone administered over a 4-day period, with subcutaneous injection once each day which will cause a mean weight increase of 50 per cent in the thyroids of 20 chicks weighing an average of 55 ± 10 gm." (p. 664), and a female unit which gives a "mean weight increase of 20 per cent" in a similarly treated group of females. One chick unit

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is equal to 0.6 mg. of their extract and is thus one-half the amount of extract utilized by Smelser to achieve an increase of 100 per cent. Fraenkel-Conrat et al. (7) base their unit on a 33 per cent increase in the thyroid weights of the experimental chicks over those of the controls in 6 days, and in their preparation 0.011 mg. contained 1 chick unit. Jensen and Grattan (13) are the only investigators who have defined a unit of thyrotropic hormone based on the histological reaction of the chick thyroid. "A chick unit is defined as the total amount of hormone which, when injected subcutaneously into 4 1-day-old chicks, twice daily for 4 days, will show a minimal stimulation of the thyroid gland by the fifth day after the onset of injection" (p. 271). However they do not describe "minimal stimulation" and give no method of measuring it.

In the present experiments, chicks have been used as hosts for anterior pituitaries from frogs (*Rana pipiens*) and mice. The aims of the tests were: *a*), to see if the chick thyroid would react to administration of pituitaries of these animals from different phyla especially in view of the possible 'species specificity' of hormones, and *b*), to compare the reaction of the chick thyroid with that already obtained in the guinea pig with pituitaries from the same donors (17, 18).

MATERIALS AND METHODS

The chicks used in these experiments were New Hampshires. They were received the day after hatching and were thus one day old when the experiments began. Upon arrival they were weighed, placed in a brooder kept at about 100° F., and fed Kerr's Starting Mash. The dish containing the food was removed from the brooder the evening before the day on which the chicks were to be killed so that their crops would be empty when they were weighed at the end of the experiment. An adequate supply of water was always available. Each chick was marked for identification on head or wings with colored paint. The sex of the chicks was determined at the end of each series when the gonads were observed.

All frog pituitaries were taken from mature female *Rana pipiens* in the interbreeding season and the mice pituitaries from adult male albino mice to correspond with other experiments carried out previously (17–19). The anterior lobe was separated from the remainder of the pituitary and weighed on a precision balance. In order to insert the gland subcutaneously the down on one side of the breast of the chick was moistened and separated to expose the skin. A hole made with a needle was enlarged with a small pointed knife. Then, under a binocular microscope, the graft was slipped through this hole on the point of a needle and was pushed back under the skin. The opening was sealed with 'New Skin.'

In each series one chick was killed on arrival (table 1; 1-day-old young). Several received injections of one frog anterior pituitary per day for one day (*series 2D*), for 2 days (*series 3C-D*), for 3 days (*series 4C-D*), for 4 days (*series 5C*), for 5 days (*series 6A*) or 2 frog anterior pituitaries per day for 4 days (*series 5B*) or for 5 days (*series 6B*). In the experiments with mouse implants, the chicks were given one or one plus mouse anterior pituitaries per day for one day (*series 2E* and *2F*) or one per day for 3 days (*series 4F*) or for 5 days (*series 6E*). One, 2, or 3 controls were given pieces of brain weighing approximately the average of the anterior pituitary substance implanted on that day (table 2). One or more untreated normal chicks were also included in each series at autopsy and the data on these were averaged with the controls (table 2). In all instances the chicks were killed with ether approximately 24 hours after the last injection and the digit with the series letter indicates the age in days of the chicks at autopsy.

The thyroids were dissected out by the method of Smelser (3) and Cope (4),

weighed on a precision balance and fixed in Helly's fluid. One gland from each chick was sectioned at 5μ. Half of the sections were stained in Mallory's connective tissue stain modified to include picro-aceto-formol as a mordant and Mayer's hematoxylin as a nuclear stain; the remainder were stained in Heidenhain's iron hematoxylin. The heights of the highest and the lowest cells in 50 follicles in a middle section of each gland were measured at 1000 diameters with a Leitz ocular micrometer. This method is a modification of that of Rawson and Starr (20) and Rawson and Salter (8) and similar to that used by Adams and Allen (18) in the guinea pig thyroid.

OBSERVATIONS

Thyroid glands of normal chicks, 1 to 6 days old. The thyroid glands of normal chicks between 1 and 6 days old appear to be very similar in their histology. They contain large follicles which are filled with colloid; the colloid varies somewhat in its content of vacuoles and in its staining capacity, although predominantly orange in Mallory's connective tissue stain; the follicles are lined with flat epithelial cells. The variation in cell height is relatively slight (table 1) and the average for the 35 chick

TABLE 1. AVERAGE BODY AND THYROID WEIGHTS AND THYROID FOLLICULAR CELL HEIGHTS IN NORMAL CHICKS 1 TO 6 DAYS OLD

Age Days	Number of Chicks	Body Weight, Autopsy Average, Range, grams	Thyroid Weight, Absolute Average, Range, milligrams	Thyroid Weight, Relative[1] Average, Range	Cell Height, Average Range, μ
1	9	39.17 32.79–51.8	3.7 1.88–5.4	0.0096 0.0045–0.0146	1.631 0.92 –2.61
2	7	39.0 31.0 –43.6	2.94 1.90–4.35	0.0075 0.0046–0.0100	1.888 1.21 –2.490
3	4	46.38 44.3 –50.7	4.02 2.61–5.50	0.0087 0.0059–0.0124	1.661 1.228–2.291
4	5	46.0 41.2 –56.2	4.35 2.32–5.87	0.0096 0.0051–0.0138	1.356 1.124–1.769
5	4	44.81 40.80–49.78	2.77 2.06–3.55	0.0063 0.0041–0.0087	1.701 1.402–2.252
6	6	48.66 42.5 –57.9	3.34 2.93–4.30	0.0070 0.0052–0.0099	1.983 1.335–2.462
1–6 Av.	35	43.21 31.0 –57.9	3.51 1.88–5.87	0.0083 0.0041–0.0146	1.715 0.921–2.490

[1] Thyroid weight as percentage of body weight.

thyroids measured in this age group is 1.715μ and very near that for nine 1-day old chicks (1.631μ), four 5-day-old ones (1.701μ) and a little less than that for six 6-day-old ones (1.983μ). In average absolute weight, there is relatively little difference for the 6-day period. At 1 day, the average absolute weight of 9 thyroids was 3.70 mg.; that for the 35 chicks 1 to 6 days old was 3.51 mg. The relative thyroid weights (thyroid weight as a percentage of body weight) vary somewhat, but at 5 and 6 days they are lower than at 1 day as body weights increase relatively faster than thyroid weights.

Chicks treated with frog anterior pituitary. The most striking changes which occurred in the chick thyroids following implantation of frog anterior pituitary com-

pared with the condition in controls receiving frog brain or in normal chicks were the histological ones (table 2; fig. 1; compare fig. 3 and 4; 5, 6, and 7). The cells became higher, the colloid more vacuolated and more blue-staining in Mallory's stain and it was decreased in amount. With a dose of one frog anterior pituitary (1.53 mg.; fig. 3 compared with fig. 4), the stimulation was evident. In this group (*series 2D*; 1 frog anterior pituitary, average dose, 1.13 mg., with autopsy 24 hours after treatment)

TABLE 2. TOTAL AMOUNTS OF FROG OR MOUSE ANTERIOR PITUITARY AND FROG OR MOUSE BRAIN ADMINISTERED IN DAILY DOSES TO YOUNG CHICKS; averages and ranges of body and thyroid weights and thyroid cell heights of the chicks. Treatment was begun when chicks were one day old. Figure in Series number indicates age in days of chick at autopsy. Controls (C) received brain; normals (N) were untreated. AP=anterior pituitary; THY=thyroid.

Series	Number of Chicks		Average Total Dosage			Average Body Weight gram		Average Thyroid Weight[1]		Av. Thy Cell Height, μ (200 Cells)	Av. % Increase in Experiment over Control Chicks		
	Experiment	Control (C) and Normal (N)	Number	AP, weight, mg.	Brain, weight, mg.	Initial	Autopsy	Absolute mg.	Relative (% of body weight)		Thyroid weight		Cell height
											Absolute	Relative	
Frog implants													
2D	8	4 (2C, 2N)	1	1.13	1.26	38.26 36.38	39.70 36.52	3.53 3.83	0.0089 0.0103	3.740 1.411	−7.8	−13.6	165.1
3C-D	8	6 (4C, 2N)	2	2.39	2.47	39.74 —	42.33 42.23	3.25 5.27	0.0077 0.0127	4.188 1.238	−38.3	−39.4	238.3
4C-D	7	6 (4C, 2N)	3	3.16	3.32	39.07 —	42.32 44.24	4.13 3.78	0.0098 0.0085	4.569 1.282	9.3	15.3	256.4
5C	5	4 (2C, 2N)	4	4.61	4.83	37.39 —	44.11 45.73	3.64 3.08	0.0083 0.0068	4.944 1.567	18.2	22.1	215.5
6A	9	5 (2C, 3N)	5	4.65	5.27	41.43 —	55.81 51.11	4.27 2.88	0.0078 0.0057	4.889 2.140	48.3	36.8	128.4
5B	6	5 (3C, 2N)	8	9.78	9.56	39.23 40.24	41.58 46.74	4.24 2.37	0.0103 0.0051	5.915 1.812	78.9	102.0	226.4
6B	7	3 (1C, 2N)	10	12.32	11.25	39.29 40.39	48.30 47.21	4.77 3.33	0.0099 0.0071	5.952 1.576	43.2	39.4	277.7
Mouse implants													
2E	7	3 (2C, 1N)	1	0.55	0.65	38.74 38.60	39.29 41.63	3.63 2.90	0.0092 0.0070	3.966 1.768	25.2	31.4	124.4
2F	8	3 (1C, 2N)	1+	1.28	1.23	37.69 37.25	40.36 39.95	6.81 2.85	0.0165 0.0071	3.260 1.863	138.6	132.4	75.0
4F	8	4 (2C, 2N)	3	3.31	3.29	37.54 37.93	38.27 42.90	4.95 4.61	0.0127 0.0107	3.655 1.556	7.4	18.7	134.9
6E	8	3 (2C, 1N)	5	4.45	4.65	38.40 37.07	40.17 42.95	3.11 2.53	0.0079 0.0059	4.714 1.666	22.9	33.9	182.9

[1] For ranges, see table 3.

there was no overlapping of the range of cell height in the experimental chicks with that of the control-normal animals (table 3) and the percentage increase in cell height was 165.1 per cent (fig. 1; table 2). With longer treatment, either with 1 anterior pituitary per day for 2, 3, 4, 5 days (fig. 5) or with 2 anterior pituitaries per day for 4 or 5 days (fig. 6) and autopsy 24 hours after the last treatment, the increases surpassed 200 per cent with one exception (*series 6A*). Here though the actual cell height was 4.889μ, practically the same as the height for *series 5C* which received almost the same average milligram dosage but in 4 instead of 5 days (table 2; fig. 1), the percentage increase was the least secured, probably because the average cell height for the control-normal thyroids was unusually high (2.140μ) due to one untreated normal and one brain-injected control which had somewhat more active glands than usual.

In *series 6B*, the most responsive gland (fig. 6) presented a very cellular picture with the colloid almost gone.

The changes in the absolute and the relative thyroid weights in anterior pituitary-treated chicks were less marked and more variable than those in cell height (table 2; fig. 1). In only one case (*series 5B*) did the percentage increase of relative thyroid weight over that of normal-control glands exceed 100 per cent and the highest percentage increase for the average absolute weight (78.9%) obtained in any series was also in this one. It is evident (table 3) that the ranges of the absolute and relative thyroid weights for control-normal and for experimental chicks overlapped in every instance and in *series 4C-D*, the ranges for the anterior pituitary-treated chicks lay wholly

TABLE 3. RANGES OF ABSOLUTE AND RELATIVE THYROID WEIGHTS AND OF CELL HEIGHTS IN CHICKS GIVEN FROG OR MOUSE ANTERIOR PITUITARY AND IN CONTROLS GIVEN FROG OR MOUSE BRAIN AND UNTREATED NORMALS.

Series	Thyroid Weights				Thyroid Cell Heights	
	Absolute, milligrams		Relative, per cent		Control-normal	Experimental
	Control-normal	Experimental	Control-normal	Experimental		
Frog implants						
2D	2.2–7.6	2.5–4.3	0.0066–0.0193	0.0059–0.0111	1.21–1.66	3.11–4.19
3C-D	3.8–7.0	1.8–4.8	0.0092–0.0172	0.0047–0.0129	1.02–1.53	3.28–4.84
4C-D	2.0–5.9	3.2–5.0	0.0052–0.0138	0.0082–0.0132	1.12–1.61	3.15–6.33
5C	2.5–3.6	3.1–4.5	0.0054–0.0087	0.0072–0.0100	1.40–1.74	4.05–5.55
6A	2.4–3.4	2.7–6.0	0.0049–0.0079	0.0047–0.0119	1.81–2.66	3.90–6.09
5B	1.9–2.9	2.8–5.6	0.0041–0.0070	0.0067–0.0144	1.43–2.25	4.29–8.74
6B	2.9–4.3	2.7–8.6	0.0046–0.0099	0.0059–0.0190	1.34–2.02	4.02–7.87
Mouse implants						
2E	1.9–4.1	1.5–4.7	0.0046–0.0104	0.0046–0.0120	1.31–2.20	1.92–5.15
2F	2.1–3.4	4.0–13.0	0.0053–0.0088	0.0093–0.0294	1.50–2.38	2.40–4.16
4F	2.3–7.60	1.7–7.5	0.0051–0.0161	0.0055–0.0184	1.28–2.06	2.13–5.42
6E	2.1–2.9	1.3–4.9	0.0055–0.0066	0.0034–0.0129	1.34–2.31	4.02–5.86

[1] Thyroid weight as percentage of body weight.

within those for the control-normal group. It is also apparent that in *series 2D* and *3C-D*, with small doses of 1 or 2 anterior pituitaries and autopsy on the 2nd and 3rd days after the first treatment, respectively, that the absolute thyroid weights of the control-normal groups exceeded those of the experimental chicks (table 2) and that the upper limits of the ranges of the control-normal groups in these two series and in *4C-D* as well were considerably higher than the upper limits for the experimental chicks in these series (table 3). It should be noted too in *series 6B* (table 2) that the highest average dose of frog anterior pituitary administered (10 glands; 12.32 mg. in 5 days with autopsy on the 6th) did not give greater percentage increases of the absolute and relative weights of the thyroids over those in control-normal chicks than those secured in *series 5B* where treatment was with 2 anterior pituitaries for 4 days with autopsy on the 5th day.

Chicks treated with mouse anterior pituitary. In the 1-day-old chicks given mouse anterior pituitary tissue and killed 24 hours after the last treatment, *i.e.*, when 2, 4 and 6 days old, histological and weight changes in the thyroids were positive in every series (table 2; compare fig. 8 and 9 with fig. 4). The cells had become markedly cu-

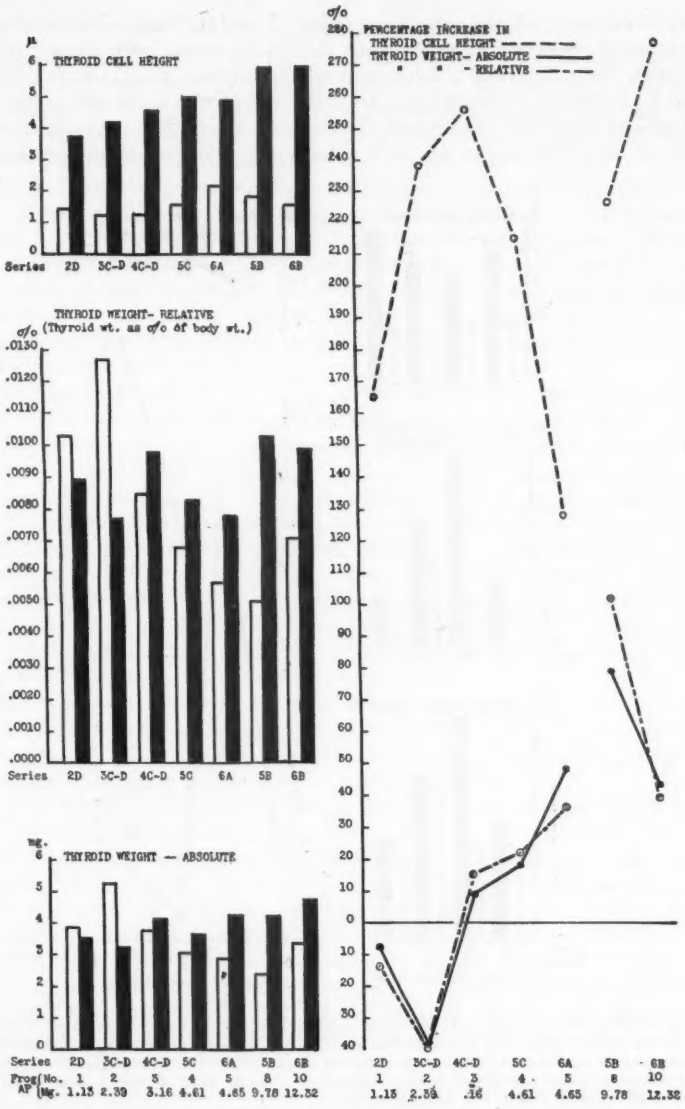
μ
THYROID CELL HEIGHT
Series 2D 3C-D 4C-D 5C 6A 5B 6B
THYROID WEIGHT- RELATIVE
o/o (Thyroid wt. as o/o of body wt.)
Series 2D 3C-D 4C-D 5C 6A 5B 6B
mg.
THYROID WEIGHT — ABSOLUTE
Series 2D 3C-D 4C-D 5C 6A 5B 6B
Frog No. 1 2 3 4 5 8 10
AP Mg. 1.15 2.39 3.16 4.61 4.65 9.78 12.32
o/o
PERCENTAGE INCREASE IN
THYROID CELL HEIGHT
THYROID WEIGHT- ABSOLUTE
" " - RELATIVE
2D 3C-D 4C-D 5C 6A 5B 6B
1 2 3 4 5 8 10
1.15 2.39 .16 4.61 4.65 9.78 12.32

Fig. 1. Graphs showing thyroid cell heights, absolute and relative thyroid weights of chicks; *solid block*, chicks given varying doses of frog anterior pituitary; hollow squares, chicks given frog brain or left untreated (control-normals). Percentage increases in experimental over control-normal groups are represented as follows: thyroid cell height, *broken line*; absolute thyroid weight, *solid line*; relative thyroid weight, *uneven dashes*. For data, see table 2.

boidal compared with the flat ones in controls and colloid was decreased in amount. The cell heights revealed average percentage increases of more than 100 per cent in

every series except *2F* and only in one series (*2E*) did the range of cell height in the experimental group overlap very slightly that in the normal-control group (table 3). Although the increases in absolute and relative thyroid weights were all definite (table 2), they did not always occur in a consistent pattern in relation to size of dose or length of treatment. For example the smallest average dose (0.55 mg.; 1 anterior pituitary; *series 2E*) in one day with autopsy the next gave almost the same per-

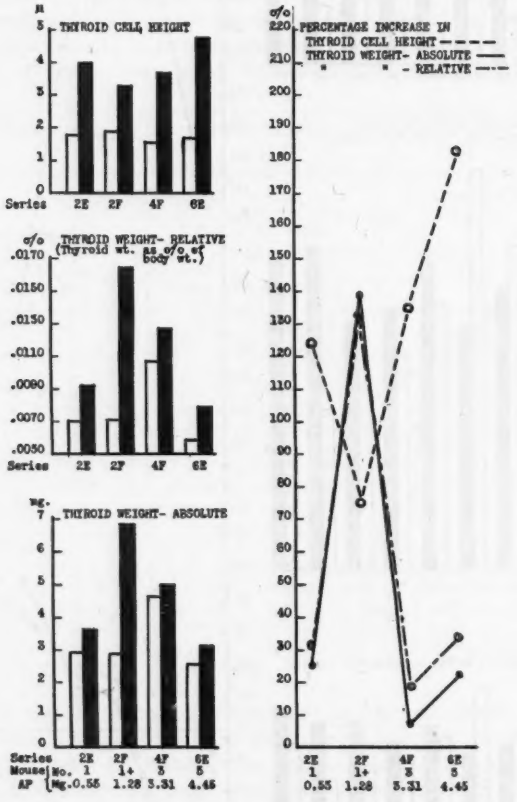

Fig. 2. Graphs showing thyroid cell heights, absolute and relative thyroid weights of chicks, *solid blocks*, chicks given varying doses of mouse anterior pituitary, *hollow squares*, chicks given mouse brain or left untreated (control-normals). Percentage increases in experimental over control-normal groups are represented as follows: thyroid cell height, *broken line;* absolute thyroid weight, *solid line;* relative thyroid weight, *uneven dashes*. For data, see table 2.

centage increases in absolute and relative thyroid weights as the largest total average dose (4.45 mg.; 1 anterior pituitary per day for 5 days with autopsy on the 6th; *series 6E;* table 2). And the greatest average percentage increases in absolute and relative thyroid weights (table 2; fig. 2) and the only instance in which the ranges for experimental and control-normal groups did not overlap (table 3) occurred in *series 2F* where the average percentage increase in cell height was the least but where the ranges of cell heights for the experimental and control-normal groups did not overlap.

DISCUSSION

The results secured indicate that chick thyroids will respond to the administration of both frog and mouse anterior pituitary (table 2; fig. 1, 2). The reaction, as judged by the increase in thyroid cell height is more pronounced and consistent than when judged by increases in absolute and relative thyroid weights. The former always occurred, even with the smallest dosage and the shortest treatment (1 anterior pituitary for 1 day with autopsy 24 hours after implantation) and with one exception (*series 2E*; table 3) the range of cell height in the experimental and control-normal groups of the 11 series did not overlap. In the experiments with frog anterior pituitary the average percentage increases were always more than 100 per cent and in 5 of the groups, more than 200 per cent. In those treated with mouse anterior pituitary they were above 100 per cent except in *series 2F* (table 2) where the figure was 75 per cent, but even here the range of thyroid cell height in experimental and control-normal groups did not overlap (table 3).

In contrast to the reaction in thyroid cell height that in thyroid weights is more variable and bears no consistent relation to the dosage given or to the length of treatment. For example, more pronounced average percentage increases in both absolute and relative thyroid weights occurred with 2 anterior pituitaries per day for 4 days (total dose 9.78 mg.; *series 5B*) than for 5 days (total dose 12.32 mg.; *series 6B*), although the absolute thyroid weight itself is greater in *series 6B* (table 2). In *series 6A* and *6B*, the percentage increases in both weights are very similar although the total dose of anterior pituitary in the former is 4.65 mg. and in the latter 12.32 mg. given in the same 5-day period. A somewhat similar situation exists in *series 2E* and *6E* given doses of 0.55 mg. and 4.45 mg. of mouse anterior pituitary in 1 and 5 days, respectively. And in *series 2D* and *3C-D*, the thyroid weights in the experimental groups are less than those in the control-normal groups.

These data make somewhat questionable the value of increases in absolute or relative thyroid weights as the best criterion for judging the thyrotropic potency of anterior pituitary substance. If one used the doubling of absolute thyroid weight as the response on which to base a unit of thyrotropic hormone (3–5) only in *series F* (table 2) in the present experiments would that have been attained. Even if lesser figures, for example increases of 50 per cent (6) or of 33 per cent (7) were used, in only three of the frog anterior pituitary-treated series (*6A*, *5B*, *6B*) and in only one of the mouse anterior pituitary-treated ones (*2F*) would an adequate response have been secured. Moreover, in normal thyroids which may weigh more than usual, the actual cell height may be very similar or even less than the average for the age-group. The heaviest thyroid in the 1-day-old group weighed 5.43 mg. and its cell height was 1.666μ as compared with 1.631μ for its group; in the 3-day-old group a gland weighing 5.50 mg. had an average cell height of 1.228μ as compared with 1.661μ for the group; in the 4-day-old group, an average cell height of 1.124μ was found in a gland weighing 5.87 mg.

An additional advantage in the use of cell-height increases in judging thyroid response is that a single small dose of anterior pituitary substance with autopsy 24 hours after administration produces pronounced effects whereas the reaction in thyroid weights may be absent or relatively small (*series 2D* and *2E*). While several investigators (3, 6, 8, 20) have recognized the importance of cell height changes after anterior pituitary administration and some (8, 20) have measured thyrotropic potency by means of it and discussed it in terms of the guinea pig thyroid weight units of Junkmann and Schoeller (21), only Jensen and Grattan (13) have defined a unit of substance based on histological grounds. Their unit is the total amount injected sub-

cutaneously into 4 1-day-old chicks which will produce a 'minimal stimulation' of the thyroid gland as determined histologically on the fifth day after two daily injections for 4 days, but what 'minimal stimulation' means in qualitative or quantitative terms is not stated. From the data of the present study a 100 per cent increase in thyroid cell height after a single dose of anterior pituitary substance with autopsy 24 hours after administration could well be used as such a unit. On this basis, less than 1 frog anterior pituitary or 1 mouse anterior pituitary, *i.e.*, less than 1 mg. of fresh substance in each case, would contain a unit. The shorter period of treatment compared with that ordinarily used in cell height measurements (8, 13, 20), and in thyroid weight changes (3–7) and in thyroid-iodine determinations (7, 10, 16) would have great advantages. It would be in line with early (1, 2) and later (22, 23) reports of the speedy reaction of the guinea pig thyroid to anterior pituitary-stimulation. Starr and Metcoff (23) have found that the thyroid cell height of anterior pituitary-treated[1] guinea pigs reached a 90.8 per cent increase over that of controls in 24 hours. Even at 16 hours, the response was 89.7 per cent and at 1, 4, 6, 8 and 12 hours, increases of 15.3, 20.0, 20.7, 70.1 and 73.9 per cent, respectively, had occurred. With autopsy later than 24 hours percentages decreased. A similar pronounced response in the sparrow thyroid at 12 hours after the injection of 0.3 gm. equivalent of thyrotropin has also been reported (24). It is also questionable whether longer periods of treatment by which doses are increased are of considerably greater value for while the response is often larger, though not always (*series* 5C, 6A, 5B; table 2), it does not necessarily bear a consistent relationship to the length of treatment or to the size of the dose and is not proportionately greater than that secured with the single treatment.

A comparison of normal thyroids of chicks, guinea pigs and mice confirms the contention that the chick thyroid has certain features, particularly its uniformly flattened epithelium, that make it a more valuable assay organ than those of the other two (3, 4, 25–27). This is especially clear when figures for the cell heights in the three animals are compared. In nine 1-day-old chicks which would correspond to the age at the beginning of the experiment, the average cell height was 1.631μ (range: 0.921–2.610μ) and in those of six 6-day-old ones, corresponding to the day of autopsy in the longest period of treatment, it was 1.983μ (range, 1.335–2.462μ; table 1). In thyroids of 11 normal guinea pigs (18) the average height was 3.134μ (range 2.373–3.910μ) and in 2 controls used in another experiment (17) it was 5.465μ (range 4.925–6.005μ). In thyroids of 8 control female mice, 22 to 23 days old at autopsy, the average thyroid cell height was even greater, 6.267μ (range, 4.24–7.10μ) (19). These figures argue both for the greater inactivity of the chick thyroid and for the greater value of relatively small increases in absolute figures which may occur in this form after anterior pituitary treatment.

On the basis of a greater uniformity of absolute thyroid weights in very young chicks, the chick has also been preferred to the guinea pig (3, 5) for assay of thyrotropic substance. Weights of thyroids in normal guinea pigs 30 days old may be extremely variable (3) and in a group of 11, 2 to 4 weeks old at autopsy, a range of 17.38 to 33.56 mg. in thyroid weights was encountered (18). In the present experiments, however, fairly wide ranges were also found in normal chick thyroid weights: 1.88 to 5.43 in 1-day-old ones and 2.93 to 4.30 mg. in 6-day-old ones. Such variations even in chick material seem to be another argument against using weight changes as a criterion for thyrotropic hormone assay.

Smelser (3) has also claimed a greater sensitivity of the chick thyroid over that of

[1] The anterior pituitary substance (Antuitrin-T) was supplied by Parke-Davis & Co., Detroit, Mich.

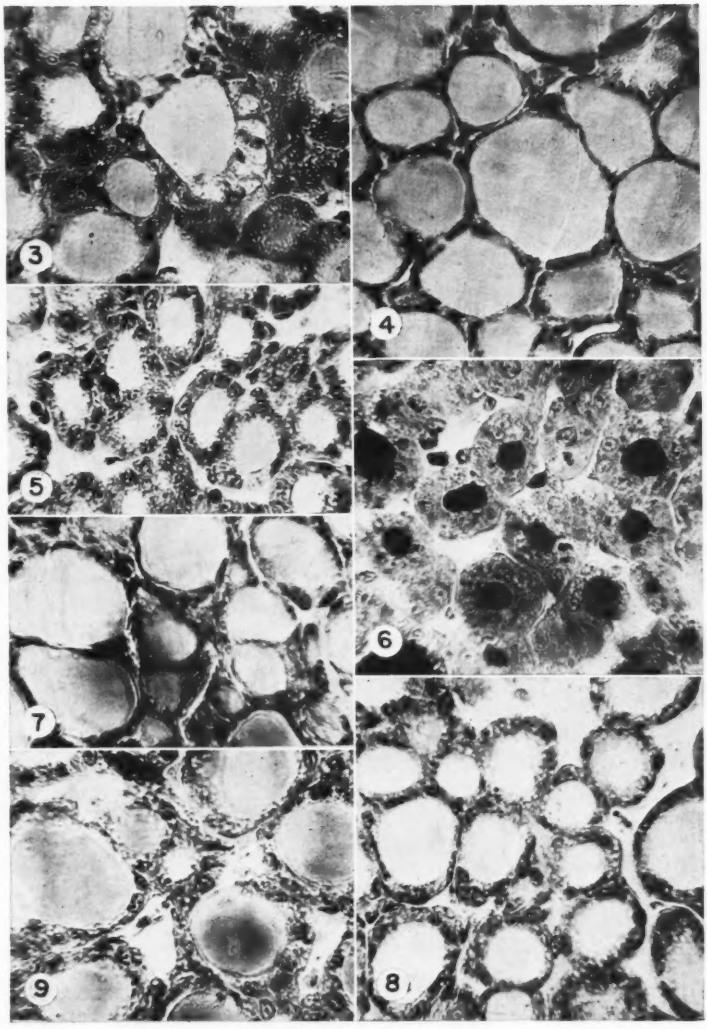

Fig. 3–9. SECTIONS OF CHICK THYROID GLANDS. Fig. 3 *Chick 83E, series 2D*, received 1 frog AP (1.5 mg.) and killed 24 hours later. Note cuboidal cells (average height, 3.96μ), round nuclei, colloid droplets in follicular cells, vacuoles in colloid. ×585. Fig. 4. *Chick 89C, series 2D*, received 1.4 mg. of frog brain and killed 24 hours later. Note flattened epithelium (average height, 1.28μ), oval nuclei, large amount of stored colloid. ×585. Fig. 5. *Chick 19E, series 6A*, received AP from 5 frogs (4.8 mg.) in 5 days and killed on the 6th day. Note cuboidal cells (average height. 5.13μ), and rounded nuclei. ×585. Fig. 6. *Chick 33E, series 6B*, received AP from 10 frogs (10.1 mg.), 2 glands per day for 5 days, and killed on the 6th day. Note very high cells (average height, 7.87μ), cell undergoing mitosis in center follicle, small amount of colloid. ×585. Fig. 7. *Chick 35C, series 6B*, received 11.3 mg. of frog brain, one-fifth of the total dose each day for 5 days, and killed on the 6th day. Note flat cells (average height, 1.38μ) and large amount of colloid. Compare with figure 6. ×585. Fig. 8. *Chick 107E, series 2E*, received 1 mouse AP (0.4 mg.) and killed 24 hours later. Note cuboidal cells (average height, 3.92μ) and colloid droplets in cytoplasm. ×585. Fig. 9. *Chick 130E, series 2F*, received 1.4 mg. of mouse AP and killed 24 hours later. Note cuboidal cells (average height, 4.05μ) and round nuclei. ×585.

the guinea pig in that the former will react by an increase in weight to one-tenth of the amount of material necessary to induce this change in the guinea pig gland. He states that "the assay range based on chick thyroid weight is much longer with a maximum response obtained with 100 times the minimal stimulating dose, whereas the maximum response of the guinea pig thyroid was obtained with only 8 times the minimum," (p. 435). Kabac and Liapin (5) have also reported that "the chick is approximately four times more sensitive to the thyrotropic extract than the guinea-pig" (p. 334). Bergman and Turner (6) have interpreted the degree of reaction somewhat differently and have stated that "on an equal body weight basis, the male chick is only slightly more sensitive to the thyrotropic hormone than the guinea pig" (footnote, p. 663). Cope (4) has also found that the dose of thyrotropic hormone per gram of body weight is approximately the same in the two hosts, but obviously since the chick body weight is so much less than that of the guinea pig, this difference results in economy of material tested.

Data secured from experiments carried on in this laboratory when examined from either of these points of view favor the use of chick rather than guinea pig or mouse for

TABLE 4. PERCENTAGE INCREASES IN ABSOLUTE AND RELATIVE THYROID WEIGHTS AND IN THYROID CELL HEIGHTS IN GUINEA PIG, CHICK AND MOUSE FOLLOWING ADMINISTRATION OF ANTERIOR PITUITARIES OF FROGS AND MICE IN 4 OR 5 DAYS OVER CONTROL FIGURES.

Group	Donor of AP	Dose of AP mg.	Host	Percentage Increases			Reference
				Thyroid Weight		Thyroid cell height	
				Absolute	Relative		
I	frog	9.78	chick	78.9	102.0	226.4	Present study
	frog	8.8	guinea pig	66.7	127.2	67.0	Adams, Granger, Rhoades (17)
II	mouse	4.45	chick	23.7	33.9	182.9	Present study
	mouse	4.90	guinea pig	1.7	7.1	20.1	Adams and Allen (18)
III	frog	12.32	chick	43.2	39.4	277.7	Present study
	frog	16.2	mouse	74.0	87.0	51.9	Adams and Tukey (19)
	frog	34.4	mouse	159.4	166.7	66.6	Adams and Tukey (19)
IV	frog	36.24	guinea pig	91.9	102.1	214.6	Adams, Granger, Rhoades (17)
	frog	34.4	mouse	159.4	166.7	66.6	Adams and Tukey (19)
V	mouse	4.45	chick	23.7	33.9	182.9	Present study
	mouse	15.19	guinea pig	30.7	6.5	69.2	Adams and Allen (18)
	mouse	25.67	guinea pig	34.3	40.0	179.2	Adams and Allen (18)
VI	frog	12.32	chick	43.2	39.4	277.7	Present study
	frog	36.24	guinea pig	91.9	102.1	214.6	Adams, Granger, Rhoades (17)

thyrotropic potency tests, provided cell height figures are used (table 4). It is evident that in *Groups I* and *II* the chick thyroid responds with a greater percentage increase in cell height than does that of the guinea pig to somewhat similar doses. In *Group III*, the chick thyroid is more responsive than that of the mouse even with smaller doses of frog anterior pituitary and in *Group IV* the guinea pig thyroid reacts more than that of the mouse to comparable doses. Thus in the order of reactivity, these three types of thyroids would stand: chick, guinea pig, mouse, Even when an adjustment is attempted by taking into account the differences in body weight, either by comparing the effects of large and small doses or by figuring what the percentage increases should have been with such corrections, the same relationship holds. For example, 15.19 mg. of mouse anterior pituitary gave a smaller increase in thyroid cell

height of the guinea pig than one-third of that amount (4.45 mg.) gave in the chick when it should have given approximately the same increase (table 4, *Group V*). A similar comparison with the 25.67 mg. dosage favors the chick thyroid also. And 12.32 mg. of frog anterior pituitary produced a greater reaction in the chick than nearly three times that amount of frog anterior pituitary (36.24 mg.) in the guinea pig, again when they should have been approximately the same (table 4, *Group VI*). Or if in *Group II*, the percentage increase for the guinea pig thyroid is estimated on the basis of the reaction obtained with a dose of 4.45 mg. of mouse anterior pituitary in the chick, the figure should be something over 60 per cent instead of 20.1. If table 4 is examined to see whether the chick is better than the guinea pig with respect to thyroid weight increases, it is clear that the variations are great and do not present any consistent pattern.

It is possible to form some opinion from the data of previous and present experiments concerning the relative thyrotropic potency of frog and mouse pituitaries, although the results are not completely unequivocal. In previous studies the frog pituitary seemed more thyrotropically potent than that of the mouse when the guinea pig thyroid was used as the test gland (17, 18). In the present one, on the basis of the injection of 1 mouse anterior pituitary (*series 2E;* average dose 0.55 mg., table 2) into 1-day-old chicks it would appear that the mouse anterior pituitary contains more thyrotropin per milligram of fresh substance than that of the frog, for an increase of 124.4 per cent occurred in the average thyroid cell height and only 165.1 per cent with twice as much (1.13 mg.) frog anterior pituitary (*series 2D*). But if the data of *series 2F* and *2D* (table 2) are used, the frog anterior pituitary seems more potent. Even when the same type of anterior pituitary is used, proportional responses may not necessarily occur for with 0.55 mg. of mouse anterior pituitary, the response was 124.4 per cent while with 1.28 mg., it was 75 per cent. The same uncertainty arises if comparisons are attempted in *series 5C, 6A* and *6E* or in *series 4C–D* and *4F*. In the former the response may be considered as favoring the greater thyrotropic potency of either frog or mouse depending on which dose of frog anterior pituitary is used for comparison, whereas in the latter, the frog anterior pituitary seems definitely more potent. Part of the difficulty may lie in the fact that although the mice used in the present experiments were supposedly mature males, they came from three different sources and varied considerably in size. In this connection, it is interesting that Bates et al. (28) have recently found a marked difference in sensitivity of the thyroid in two different strains of the same breed of chicks to anterior pituitary stimulation. However, at present it would seem wiser to suspend judgment as to relative potencies of frog and mouse anterior pituitary tissue though it appears that both are high in thyrotropin.

Although Witschi (29) suggests the possibility that there may be as many gonadotropic, thyrotropic and other hormones of the anterior pituitary as there are vertebrate species, evidence against the concept of 'species specificity' of the anterior pituitary thyrotropic hormone has already been reported (18) on the basis of the guinea pig thyroid reaction to anterior pituitary administration from a variety of donors. Additional evidence is provided by the reaction of the thyroids of chicks and other birds to the thyrotropic hormone produced in the anterior pituitaries of numerous very distantly related animals. One of the first accounts of a histological reaction of hen thyroids to anterior pituitary stimulation is that of Noether (30) and this response or a weight increase or a change in thyroid-iodine content has been reported many times since in young chicks (one day to 10 weeks old) or even in embryos of 8 to 18 days of incubation following the use of mammalian (beef, sheep, hog) anterior pituitary extracts (3–8, 10–16, 31). In December, 1940, while some of the experiments of the pres-

ent study were in progress, it was reported by Keaty and Stanley (32) that 5.0 mg. of fresh anterior pituitary substance from bull frogs (*Rana catesbiana*) would induce histological changes in thyroids of two-day-old chicks, but it is not stated what these changes were in increases in cell height or whether they could be obtained with less than a 5.0 mg. dosage. In the same year Foglia (9) also published data on increased thyroid weights ranging from 11.1 to 85.0 per cent in 1-day-old chicks injected with a total of 3, 5, 10, 21 toad pituitaries (*B. arenarum*) representing doses of 9.9 to 69.3 mg. of fresh or 1.65 to 11.55 mg. of dry substance, given in three doses in one day with autopsy 24 hours after the first dose. One anterior pituitary induced no response. The thyroids of doves, pigeons, sparrows, canaries, and ducks have also responded with increases in weight and cell height to administration of mammalian (cattle, hog, sheep, human) anterior pituitary extracts (doves and pigeons: 7, 33–37; sparrows and canaries: 24, 38; ducks: 39, 40) and to fresh frog anterior pituitary substance (sparrows: 32). Such data do not support, for the thyrotropic hormone, the statement of Creaser and Gorbman (41) for gonadotropic ones "that the effectiveness of a gonadotropic hormone in a foreign species tends to vary directly with the phylogenetic proximity of the donor and recipient species" (p. 327). On such a basis, frog and toad anterior pituitary should be markedly impotent compared with mammalian anterior pituitary when tested in birds such as the chick or in mammals such as the guinea pig, mouse, or rat (9, 17-19, 42). The data may thus be interpreted in favor of the non-specificity of the thyrotropin of the vertebrate anterior pituitary gland (18), and of differences of a quantitative sort. However, the possibility of qualitative chemical differences cannot be ruled out until chemically pure substances are secured and analyzed. In this connection it is interesting that for the chemically pure lactogenic hormone of ox and sheep anterior pituitary glands which produces the same biological effect in tests, 'species specificity' has been claimed on the basis of differences in solubility and tyrosine content of the two extracts although they were indistinguishable electrophoretically and immunologically and contained similar amounts of tryptophane, arginine, sulfur, and had the same molecular weights (43, 44). Such studies present the only sound basis on which the question of 'species specificity' can be settled.

SUMMARY

Frog and mouse anterior pituitaries were implanted subcutaneously into 1-day-old chicks for periods of 1 to 5 days with autopsy 24 hours after the last treatment. Absolute weight, relative weight (gland weight as a percentage of body weight), cell height and histology of the thyroids of these chicks were compared with those in control-normal (brain-implanted-untreated) groups.

Increases in thyroid cell height of anterior pituitary-treated chicks over the figures in control-normals always occurred and with one exception (*series 2F*) were over 100 per cent. A unit of thyrotropic hormone is suggested as that contained in an amount of fresh substance which will give a 100 per cent increase in thyroid cell height of five to ten 1-day-old chicks 24 hours after subcutaneous administration in a single dose. Accompanying the heightened epithelium were significant changes in the colloid. Although increases in absolute and relative thyroid weights of the anterior pituitary-treated chicks usually occurred, they were smaller and more variable than cell height changes and in a few cases absent. Such data indicate the greater value of cell height over weight changes in thyroids as a criterion for thyrotropic hormone assay.

A comparison of the reaction of the chick and guinea pig thyroids to frog and

mouse anterior pituitaries favors the chick gland as a more sensitive indicator than that of the guinea pig for tests of thyrotropic hormone.

The data secured in the present experiments are discussed in relation to the question of species specificity of anterior pituitary hormones.

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SESAME OIL AS A VEHICLE FOR FAT SOLUBLE HORMONES[1]

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ACCUMULATING EVIDENCE indicates that sesame oil, often used as a vehicle for fat soluble hormones, is not an inert substance. Pollia (1) found that sarcomatous rats exhibited serious injury to visceral organs after intraperitoneal injections of sesame oil. Spurr and Kochakian (2) reported that this oil decreased the survival time of adrenalectomized rats by an average of 1.6 days. Tobin (3) and Bruce and Tobin (4), on the other hand, found that while sesame oil was toxic to normal rats it prolonged the survival time of adrenalectomized rats. Clauson (5) reported that this oil would cause a decrease in the size of the thymus glands of adult male rats and Stein and Allen (6) found that it increased the number of mitoses in the germinal epithelium of the mouse ovary. From these reports it seems clear that sesame oil is not always physiologically inactive and that it may act either as a stimulator or as an inhibitor depending upon the conditions of the experiment.

This present work was undertaken in an attempt to find a possible influence of sesame oil on the effectiveness of small doses of a hormone dissolved in it.

METHODS AND RESULTS

Immature male mice, 22 days of age, were used except where otherwise indicated. Androsterone was the hormone chosen and it was dissolved in sesame oil in such proportions that the desired doses could be injected in totals of 0.5, 1.0, 1.5, and 2.0 cc. of oily solution. The injections were given subcutaneously from the 22nd to the 31st day of life. Autopsies were performed on *day* 32. The prostate and seminal vesicles were removed as one mass and weighed. Sesame oil alone was given in total doses of 0.5, 1.0, 1.5, 2.0 and 3.0 cc. in exactly the same manner.

Immature mice. From a study of 58 normal immature male mice, 32 days of age, it was found that the average body weight was 14.3 gm. with a mean deviation[2] of ± 2.5 gm., and the average ratio of prostate and seminal vesicle to body weight was 2.36 with a mean deviation of ± 0.84.

Another series of 58 immature male mice, litter mates of the above controls, was injected with various amounts of androsterone dissolved in sesame oil. The detailed doses and results are shown in table 1 and figure 1. Doses of androsterone up to 200 micrograms per day for 10 days did not produce the expected stimulation of the prostate and seminal vesicles; indeed, the weight of the organs of the mice receiving the 200 μg. per day averaged 50 per cent less than that of the controls. That the hormone was active material is shown in the 5 animals given larger doses of androsterone amounting to a total of 10,000 and 25,000 μg. The ratio of prostate and

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[2] As obtained by: $E = \sqrt{\frac{\sum d^2}{N-1}}$

seminal vesicle to the body weight of these animals showed a definite increase from the control figure of 2.36 to 2.56 and 4.23.

There is no correlation between the amount of androsterone injected and the amount of decrease in prostate and seminal vesicle weight obtained. There does seem to be some correlation, however, with the amount of sesame oil used as a vehicle. Total doses of 100 and 500 μg., both given in a total of 0.5 cc. of sesame oil, were followed by less decrease in prostate and seminal vesicle weight than other doses of androsterone given in a total of 1.0, 1.5, or 2.0 cc. of oil. These results indicated that the sesame oil was responsible for this decrease, and not the androsterone.

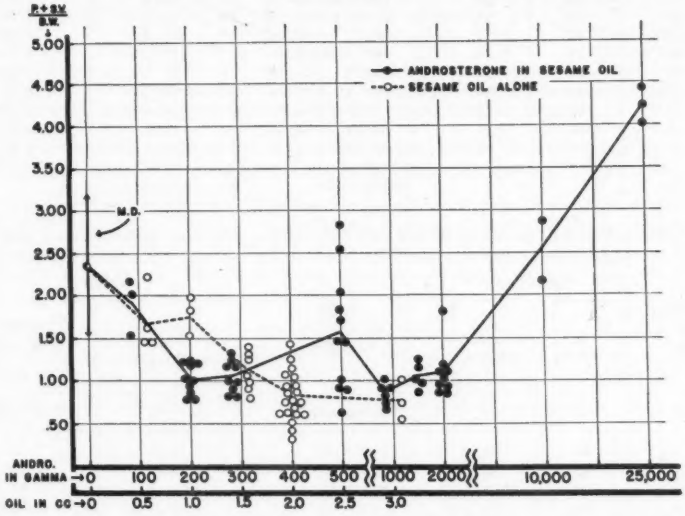

Fig. 1. Ratio of prostate-seminal vesicle weight to body weight in mice treated with androsterone.

Accordingly, 37 immature male mice of the same age were treated in an identical manner with totals of 0.5, 1.0, 1.5, 2.0, and 3.0 cc. of sesame oil alone. The details are given in table 2 and figure 1. With an increase in the amount of sesame oil there was a corresponding decrease in the prostate and seminal vesicle weight. A total dose of 3.0 cc. of oil in 10 days produced animals with an average ratio of prostate and seminal vesicle to body weight of 0.78 as compared to that for the controls of 2.36. The curves for the androsterone-treated animals and for the sesame oil-treated animals are practically identical (fig. 1).

The average body weight of the 58 androsterone-treated mice was 14.6 gm. with a mean deviation of ±2.3 gm.; the 37 sesame-oil-treated mice, 14.0 gm. with a mean deviation of ±2.5 gm. The average weight of the 58 controls was 14.3 (±2.5). gm.

Adult mice. Forty-three adult male mice were studied in an attempt to determine whether these effects were limited to the immature animal. These mice were divided into three groups according to weight; one-half of each group served as controls. The treated mice of *groups* 1 and 3 were injected with 0.2 cc. of sesame oil per day for 30 days; *group* 2, with 0.3 cc. per day for 30 days. The prostate and the seminal vesicles were weighed at autopsy. The results are tabulated in table 3. There was no decrease in the prostate and seminal vesicle weight in the adult mice comparable to that obtained in the immature mice.

TABLE 1. EFFECT OF ANDROSTERONE DISSOLVED IN SESAME OIL ON THE PROSTATE AND SEMINAL VESICLES OF IMMATURE MALE MICE

Number of Mice	Age, Days		Total Androsterone Treatment, μg.	Average	
	At start	At autopsy		Body weight, gm.	Prostate+Seminal Vesicle / Body weight
58	22	32	No treatment	14.3±2.5	2.36±.84
3	22	32	100 in 0.5 cc. oil	18.4	1.91
10	22	32	200 in 1.0 cc. oil	14.2	1.00
9	22	32	300 in 1.5 cc. oil	13.8	1.05
11	22	32	500 in 0.5 cc. oil	15.3	1.59
6	22	32	1,000 in 1.0 cc. oil	15.1	0.87
5	22	32	1,500 in 1.5 cc. oil	14.3	1.05
9	22	32	2,000 in 2.0 cc. oil	14.4	1.10
2	22	32	10,000 in 1.0 cc. oil	14.8	2.56
3	22	32	25,000 in 2.0 cc. oil	12.6	4.23
58			Treated mice	14.6±2.3	

TABLE 2. EFFECT OF SESAME OIL ON PROSTATE AND SEMINAL VESICLES OF IMMATURE MALE MICE

Number of Mice	Age, Days		Total Sesame Oil Treatment	Average	
	At start	At autopsy		Body weight, gm.	Prostate+seminal vesicle / Body weight
58	22	32	No treatment	14.3±2.5	2.36±.84
4	22	32	0.5 cc.	11.8	1.69
3	22	32	1.0 cc.	12.9	1.76
7	22	32	1.5 cc.	14.3	1.11
20	22	32	2.0 cc.	14.4	0.84
3	22	32	3.0 cc.	14.1	0.78
37			Treated mice	14.0±2.5	

DISCUSSION

Sesame oil does not seem to be an inert substance. These data show that this oil will prevent the expected effect of a given dose of androsterone when this hormone is administered in small amounts such as would be obtained in the daily urinary output of the human male. The decrease in weight obtained in the prostate and seminal vesicles was proportional to the amount of sesame oil used as a vehicle. Sesame oil alone produced the same effects as the oil and androsterone mixtures.

This response to sesame oil was much more marked in the immature mice than in the adults. The latter showed slight if any effects. This difference in response is possibly due to the greater sensitivity of developing sexual organs than of mature sexual organs to toxic agents.

Bruce and Tobin (4) found that daily intraperitoneal injections of 0.25 cc. of sesame oil in the normal adult male rat inhibited growth, increased the adrenal weight, decreased the testis weight, and slightly decreased the seminal vesicle weight after 63 days of injections. Increased doses caused the death of the animals. By means of ethyl alcohol extraction, these same workers separated sesame oil into an alcohol-soluble fraction and an alcohol-insoluble fraction. The alcohol-soluble fraction was toxic to immature adrenalectomized rats while the alcohol-insoluble fraction prolonged the survival time. The latter fraction also increased the survival time and the

TABLE 3. EFFECT OF SESAME OIL ON WEIGHT OF PROSTATE AND SEMINAL VESICLES OF ADULT MALE MICE

Group Number	Number of Mice	Sesame Oil Treatment	Average	
			Body weight, gm.	Prostate and seminal vesicle weight, gm.
1	7	None	25.2	222.4±55.6
	7	0.2 cc. oil/day×30	27.1	188.0±29.8
2	7	None	26.9	324.1±86.7
	8	0.3 cc. oil/day×30	34.3	339.2±76.1
3	7	None	28.7	302.0±58.6
	7	0.2 cc. oil/day×30	30.6	292.9±70.7

Thirty days of injections produced subcutaneous accumulations of oil which increased the weight of the treated animals. The ratio of organ weight to body weight was, therefore, not used.

lactating performance of adult rats adrenalectomized before parturition to such an extent that 47 per cent of the young were brought to weaning age. Synthetic tricaproin, synthetic alphatocopherol, wheat germ oil, pure linoleic acid and all fractions obtained from hydrolysis of sesame oil shortened the survival time of adrenalectomized rats. Buchheim (7) reported that men working in atmospheres of high concentration of oil vapors lacked sexual desire and that rats treated with 'mazout' showed a decrease in the weight of the prostate and seminal vesicles.

In order for comparisons to be made, any oil used as a solvent should be absorbed at a uniform rate. Turner and Mulliken (8) found that the rate of absorption of subcutaneous injections of corn oil varied with the strain of mice used and with the sex of the animals. The oil was absorbed more rapidly in males than females. Castration before puberty abolished this difference.

Merely because sesame oil inhibited the growth of the prostate and seminal vesicles of the immature mouse does not indicate that all organs studied will respond in a similar manner to sesame oil injections. However, with the increasing number of reports on responses to this oil, some that it is stimulating and others that it is inhibitory in nature, it is recommended that the use of large quantities as an injecting vehicle be avoided and, if possible, that it not be used in assay work. If it is necessary to use an oil solvent, hormones should be prepared for injection in such a manner that equal quantities of oil can be injected with the various doses of the stimulating agent.

SUMMARY

1. Sesame oil may prevent the expected effect of small amounts of a hormone dissolved in it. Injections of small doses of androsterone dissolved in sesame oil into immature male mice for 10 days were followed by a reduction in weight of the prostate and seminal vesicles.

2. Subcutaneous injections of sesame oil alone produced a similar decrease in the weight of the prostate and seminal vesicles of immature male mice.

3. Sesame oil did not produce a significant change in the weight of the prostate and seminal vesicles of adult mice treated for 30 days with 0.2 and 0.3 cc. per day.

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NOTES AND COMMENTS

EFFECT OF SHEEP ANTERIOR PITUITARY EXTRACT ON RABBIT OVARIES AND UTERUS

THE PURPOSE of this study was to determine the effect of sheep pituitary gonadotropin* on the ovaries and uterus of normal, bilaterally, and unilaterally oöphorectomized and hysterectomized rabbits.

The method followed was to inject animals twice daily for 3 days with a total of 10 R.U. of pituitary extract and biopsy on the 6th day. The extract was freshly prepared for each injection by dissolving an amount of powder in saline solution so that 6 injections of 0.1 cc. each would contain a total of 10 R.U.

Four control rabbits were treated as above and opened on the 6th day, at which time one ovary was removed and a biopsy of the uterus made. In 3 of 4 animals a 4 + endometrial proliferation was obtained, and recent corpora lutea and active follicles found both macroscopically and microscopically.[1] In the fourth animal a 2 to 3 + endometrial proliferation was noted along with corpora lutea and active follicles.

One ovary was removed from each of a group of 6 animals and injections made. Biopsy and ovarian sections on these animals showed 1 to 2 +, 2 to 3 +, 3 to 4 +, 3 to 4 +, 4 + and 4 +, respectively, with greatly hypertrophied ovaries in each case and corpora lutea and follicles. The hypertrophy of the ovary was due to an increase of the stroma, the corpora lutea and follicular activity being about normal.

Both ovaries were removed from 4 animals and the procedure repeated. In no case could any trace of endometrial proliferation be shown. In fact the endometrium had become atrophic and the uterus was grossly very much smaller than normal.

Four animals, with the uterus removed, were injected and the ovaries showed early corpora lutea and follicular activity not unlike that seen in the normal group, both macroscopically and microscopically.

Conclusions. In a normal animal there seems to be a gradation of changes possible after the injection of anterior pituitary extract. However, on the removal of one ovary, the remaining gonad became hypertrophied, and a marked decrease in endometrial proliferation resulted. Gynecological supposition holds that in hypogonadal cases with one ovary removed the pituitary will overstimulate the remaining ovary and hence result in more nearly normal ovarian and uterine function. The results in the rabbit indicate that this conception is false.

With both ovaries removed no endometrial activity could be demonstrated, in fact atrophic changes were noted. However, when the uterus is removed, injection of the anterior pituitary extract still produces a change in the ovaries of a quality equal to that produced in the normal animals.

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* The gonadotropin (Preloban) is a purified sheep pituitary powder supplied by Winthrop Chemical Co., New York City.

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